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UNIVERSITY OF ALBERTA

REMOVAL OF MICROCYSTIN-LR TOXIN FROM DRINKING WATER

by

TIMOTHY W. LAMBERT

©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Removal of Microcystin-LR Toxin from Drinking Water** submitted by **Timothy W. Lambert** in partial fulfillment of the requirements for the degree of **Master of Science** in **Environmental Science.**

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ABSTRACT

This thesis focuses on the behaviour and identification of the cyanobacterial microcystin toxins in water treatment processes at environmentally relevant concentrations. The protein phosphatase bioassay was applied to quantify the concentration of microcystin-LR in water. The removal of microcystin by conventional processes and granular activated carbon (GAC) filtration at a small water treatment facility in Alberta was evaluated. Raw and treated concentrations of total microcystins at a second treatment facility utilizing powdered activated carbon (PAC) were determined over the month of September, 1992. Adsorption isotherms were constructed for microcystin-LR using virgin and pre-loaded carbon removed from the GAC bed at the treatment facility.

A detailed review of the toxicology and epidemiology of microcystin-LR was performed. Microcystin-LR has been shown to be a potent hepatotoxin, and liver tumor promoter in experimental animals. Case reports suggest that the microcystin toxins may have been the cause of several animal and human poisonings. Biochemical research suggests that the toxic effects of microcystin-LR may be caused by their inhibition of protein phosphatases.

Microcystin-LR standard curves for the protein phosphatase bioassay were prepared. The dependence of microcystin-LR inhibition of protein phosphatase activity on the concentration of enzyme in the standard assay was evaluated. The protein phosphatase assay was used to quantify microcystin in raw water and finished drinking water at concentrations as low as 0.1 μg/L microcystin-LR.

Microcystin-LR was purified from a water sample collected prior to GAC filtration. The identity of microcystin-LR was confirmed by chromatographic co-migration with standard microcystin-LR with two distinct conditions of high performance liquid chromatography combined with protein phosphatase inhibition measurement.

A residual microcystin concentration was found in the drinking water at both treatment facilities at all sampling times. The residual concentration was relatively constant (~ 0.1 μg/L) despite variations in the influent concentration of microcystin. There was a significant difference in virgin and pre-loaded carbon adsorption isotherms for microcystin-LR in distilled water. An adsorption isotherm conducted with virgin carbon using water containing natural organic matter was similar to the pre-loaded carbon isotherm.

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1.0 INTRODUCTION

This thesis focuses on the removal of microcystin toxins from drinking water. The microcystin toxins are produced by freshwater cyanobacteria. Cyanobacteria, also known as blue-green algae, are one of the two divisions of prokaryotes. Cyanobacteria are extremely old organisms and they are believed to have been the first colonizers of land in the course of biological evolution (Schopf 1993). The microcystin toxins are produced by the common planktonic cyanobacteria found in surface water supplies but their biological function is unknown (Carmichael 1992). The microcystins have been shown to be extremely potent hepatotoxins in a variety of experimental animals and they are lethal at high concentrations. They have also been shown to be a powerful tumor promoter (Nishiwaki-Matsushima et al. 1992, Falconer 1991). The presence of toxic cyanobacteria in surface water has been documented in all parts of the world (Namikoshi et al. 1992, Lawton and Codd 1991, Carmichael et al. 1988, Falconer et al. 1983b, Kenefick et al. 1992, Kotak et al. 1993). The microcystin toxins have been related to several incidents of animal and human illness in Canada, United States, Australia and Africa. Therefore exposure to the microcystin toxins is a potentially significant health risk and toxin removal from drinking water is a concern world wide.

1.1 BACKGROUND

Evidence of toxic cyanobacteria dates back at least to 1878, when livestock poisonings were described after consumption of water containing a cyanobacterial bloom (Francis 1878). Animal poisoning usually occurs when the bloom material becomes concentrated near the shore where the animals drink. This exposure scenario was articulated by Francis (1878):

> "Thus floating, it is wafted to the lee shores, and forming a thick scum like green oil paint, some two to six inches thick, and as thick as pasty porridge, it is swallowed by cattle when drinking, especially such as suck their drink at the surface like horses"

The toxicity of microcystin has been attributed to the inhibition of protein phosphatases (Eriksson et al. 1990). The protein phosphatases are very conserved in evolution and their properties are very similar in species as diverse as plants and mammals (MacKintosh et al. 1990). Therefore, humans may be as susceptible to microcystin toxin as other mammals. There are fewer reports of human than animal poisonings and it has been suggested that humans are repelled by the idea of using water containing algal blooms (Carmichael 1992). The notion that water is repelling if containing an algal bloom was expressed by Tolstoy (1857), in his epic novel "War and Peace". The summer was very hot, and Russia was experiencing a severe drought. The Russian troops were retreating to Moscow when they came upon a small lake;

> "As he rode along the embankment Prince Andrei smelt the fresh, muddy smell of the lake. He longed to take a dive into the water, however dirty it might be. .... The small lake, thickly covered with green slime, looked half a yard higher and overflowed the dam... "

However, poisoning by microcystin need not only occur by exposure to the cyanobacteria biomass. The microcystin toxins have been observed in the water surrounding the bloom material, believed to be released when the cyanobacterial cells die (Fitch et al. 1934, Steyn 1945). Microcystin toxins are also released from cells after treatment of cyanobacteria blooms with copper sulphate (Kenefick et al. 1993, Prescott 1948). Copper sulphate has been used as a chemical agent to control toxic blooms since 1904 in the United States (Prescott 1948, McKnight et al. 1983). Therefore exposure to the toxins may occur by consumption of water containing the bloom material.

Early water treatment experiments conducted with crude cyanobacteria extracts determined that activated carbon is capable of removing the toxic component(s) from water (Wheeler et al. 1942, Stewart et al. 1950, Shelbusky 1951). Recent experiments conducted with toxin extracts have reached similar conclusions and have also shown that conventional processes are not that effective in removing the microcystin toxins (Falconer et al. 1983a and 1989, Himberg et al. 1989, Hoffman 1976, Kiejola et al. 1988).

1.2 SCOPE OF THE THESIS

To determine the possible health risk associated with using water sources tainted with toxic cyanobacteria, the concentration of microcystin toxin in drinking water and the effectiveness of water treatment processes in removing the toxin must be known. This research was aimed at quantifying the presence of microcystin toxin in drinking water and characterising the removal of microcystin by drinking water treatment processes at environmentally relevant concentrations.

The removal of microcystin was evaluated at two small treatment facilities where the raw water has a known history of toxic cyanobacteria (Kotak et al. 1993). One treatment facility has the conventional water treatment processes (coagulation -sedimentation, dual media filtration, and chlorination) and granular activated carbon filtration. The second facility employs the conventional treatment processes combined with powdered activated carbon. Adsorption isotherms for microcystin-LR, a very common microcystin toxin, were determined to evaluate the removal of microcystin by activated carbon. The presence and identity of microcystin-LR in the drinking water was confirmed by chromatographic comparison with standard microcystin-LR. The protein phosphatase bioassay (Holmes 1991) was applied to quantify the concentration of microcystin in the drinking water.

The thesis has been prepared in the paper format. Each chapter covers a different aspect of the thesis work and each will be submitted for publication. Chapter two is a review paper of the toxicology and epidemiology of microcystin, the water treatment experiments, and the analytical techniques available. The application of the protein phosphatase bioassay is described in chapter three. The analysis of both water treatment plants, the adsorption isotherms, and the purification of microcystin from the drinking water are presented in chapter four. The final chapters consist of conclusions integrating the previous chapters of the thesis and a discussion of future research possibilities.

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2.0 MICROCYSTIN TOXINS: HEALTH EFFECTS AND SAFETY OF DRINKING WATER SUPPLIES

2.1 OCCURRENCE OF CYANOBACTERIAL TOXINS IN WATER SUPPLIES

Cyanobacteria (blue-green algae) produce two types of toxins, neurotoxins and hepatotoxins. Both are produced by the common planktonic cyanobacterial genera found in surface water supplies and have been related to several incidents of human and animal illness (Carmichael 1992). Toxic cyanobacteria blooms have been documented in Canada (Carmichael and Gorham 1981, Kotak et al. 1993), United States (Repavich et al. 1990), New Zealand, Australia, USSR, Bangladesh, India, Israel, several European countries (Skulberg et al. 1984), South Africa (Scott 1991) Japan (Shirai et al. 1991) and China (Carmichael et al. 1988). The presence of toxic blooms in drinking water sources has been documented in Finland, (Namikoshi et al. 1992), England and Europe (Lawton and Codd, 1991), China (Carmichael et al 1988), Australia (Falconer et al. 1983c), and western Canada (Kenefick et al. 1992).

Cyanobacteria blooms persist in water supplies when adequate levels of nutrients, especially phosphorus and nitrogen, are coupled with favourable environmental conditions, water temperatures between 15 and 30 °C, and a pH between 6 and 9 (Skulberg et al. 1984). Some cyanobacteria can fix atmospheric nitrogen, and low levels of nitrogen gives a nutritional advantage to these species (Gibson and Smith, 1982). Surface blooms of cyanobacteria occur under stratified conditions, when the cyanobacteria, by virtue of their buoyancy regulation via gas vesicles, stay in the photic zone at an optimum depth for photosynthesis. In contrast, the diatoms and non-flagellate green algae are unable to maintain their position in the water column and can sink below the photic zone.

Early attempts to establish correlations between toxicity and growth were conducted by Grant and Hughes (1953) with fresh cyanobacteria cells, and Hughes et al. (1958) with cultured *Microcystis aeruginosa*. Watanabe and Oishi (1983) followed the toxicity development of a cultured strain of *Microcystis aeruginosa* through lag, exponential and stationary growth phases. Maximum toxicity was observed between the exponential and stationary growth phases. Watanabe et al. (1989), found that the highest cellular content of toxins in two cultured *Microcystis* strains was at the late stage of exponential growth.

The ability to predict the toxicity of a bloom with knowledge of the environmental conditions has not been established, as variations in toxicity change over a short period of time and spatially within the bloom itself (Carmichael and Gorham 1981). Several studies have attempted to correlate environmental parameters and bloom toxicity (Sivonen 1990, Utkilen and Gjolme 1992, vander Westhuizen et al. 1986, Watanbe et al. 1989, Watanabe and Oishi, 1985). However, toxicity assessments of cyanobacteria blooms by mouse bioassay indicate that about 50% of all cyanobacteria blooms are toxic (Carmichael and Gorham 1981, Kotak et al. 1993, Lawton and Codd 1991, Repavich et al. 1990, Sivonen et al. 1990, Watanabe et al. 1991).

2.2 TOXICOLOGY OF MICROCYSTIN-LR

Microcystin-LR is perhaps the best known cyanobacteria hepatotoxin, but there are a great number of variations. The structure of microcystin-LR is shown in Figure 2.1. The general structure is cyclo(-D-Ala-L-X-D-*erythro*-β-methyl-Asp-L-Z-Adda-D-Glu-N-methyldehydro-Ala); where X and Z represent two variable L amino acids, (leucine, arginine, tyrosine, alanine, or methionine); and Adda (2S,3S,8S,9S,)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid is the unique C20 amino acid found only in these cyanobacterial peptide toxins (Botes et al. 1985). In microcystin - LR, the X is leucine and the Z is arginine. There have been several minor variations in the general structure reported (Carmichael 1992), and a D-Serine variation at the D-Ala residue (Sivonen et al. 1992).

2.2.1 Mechanism of Toxicity

2.2.1.1 Microcystin-LR Inhibits A Cellular Signal Transduction Mechanism

Microcystin-LR exerts its toxic effect by interfering with a major cellular signal transduction mechanism, reversible phosphorylation. Reversible phosphorylation is like a switch that turns biological processes on and off. An example of a biological process that is controlled by reversible phosphorylation is glycogen metabolism. Glycogen is made up of several glucose molecules linked together and serves as an energy reserve in cells. When cells need energy, the enzymes in the process are phosphorylated by a protein kinase. Phosphorylation turns on the enzyme that cleaves glucose molecules from glycogen, and turns off the enzyme that synthesizes glycogen from glucose (Figure 2.2). Conversely, when cells are storing energy, the enzymes in the process are dephosphorylated by a protein phosphatase. This results in turning off the enzyme that cleaves glucose from glycogen and turning on the enzyme that attaches glucose to glycogen. Reversible phosphorylation is used to control a wide variety of cellular processes as diverse as muscle contraction, cell division, metabolism, and memory. The specifics of each system are unique but they share the underlying mechanism of phosphorylation by protein kinases and dephosphorylation by protein phosphatases (Cohen 1989). Microcystin-LR interferes with these signal transduction mechanisms by inhibiting the function of protein phosphatases (Honkanen et al. 1990, MacKintosh et al. 1990).

2.2.1.2 Protein Phosphatases

There are a variety of protein phosphatases that dephosphorylate serine, threonine, and tyrosine amino acid residues in target proteins. Microcystin-LR inhibits only two specific types of protein phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP1 and PP2A dephosphorylate phosphoseryl or phosphothreonyl proteins. PP1 and PP2A have distinct properties, see Table 2.1 for details (Cohen 1989). PP1 binds to specific targeting subunits which localize PP1 to particular cell processes. For example, PP1 can attach to a glycogen binding subunit, called the G-subunit, which localizes PP1 to glycogen. No targetting subunits have yet been identified for PP2A and this enzyme is found mainly in the cytoplasm of eukaryotic cells. PP1 and PP2A phosphatases have broad and overlapping substrate specificities and they function conjointly to control intracellular processes by reversible phosphorylation.

2.2.1.3 Regulation of Protein Phosphatases

Protein phosphatases and protein kinases are activated/deactivated by phosphorylation and by the presence of chemical messengers whose concentration changes in response to the cells particular needs. For example, the hormone adrenaline is a chemical messenger that signals the cell to make energy, thereby activating protein kinases. PP1 is also deactivated by two endogenous inhibitors, called inhibitor-1 and inhibitor-2 (Cohen 1989). A more detailed glance at glycogen metabolism will demonstrate how protein phosphatases and protein kinases are regulated (Figure 2.3). When adrenaline signals the cells to produce energy, this causes an increase in concentration of a second messenger, cyclic AMP (cAMP), which turns on cAMP-dependent protein kinase (PKA). This results in activation of a cascade of protein kinases which phosphorylate the glycogen cleaving enzyme, glycogen phosphorylase, and the glycogen synthesizing enzyme, glycogen synthase. However, PP1 is also deactivated by phosphorylation. First the G-subunit, (glycogen binding subunit) is phosphorylated at two locations by PKA and this causes PP1 and the G-subunit to dissociate. Inhibitor-1 is also phosphorylated by PKA. This induces inhibitor-1 to bind PP1 deactivating it. Energy production is turned off and PP1 reactivated by type 2 phosphatases. PP2B cleaves the phosphate from inhibitor-1 causing inhibitor-1 to release from PP1 (Cohen and Cohen 1989). PP2A cleaves a phosphate from the G-subunit and this allows PP1 and the G-subunit to bind, and attach to glycogen (Cohen 1992). The PP1/G-subunit then dephosphorylates glycogen synthase and glycogen phosphorylase thus activating energy storage in the form of glycogen. Regulation of protein phosphatases has not been elucidated for all the cellular processes involving reversible phosphorylation but this example illustrates the general mechanisms involved.

2.2.1.4 Microcystin-LR Inhibition of Protein Phosphatases

Microcystin-LR has been shown to deactivate PP1 and PP2A by binding to their catalytic subunit (Honkanen et al. 1990, MacKintosh et al. 1990). This results in a situation where the reversible phosphorylation switch is left in the phosphorylated position. In the case of glycogen metabolism, deactivation of PP1 and PP2A would result in the release of glucose from glycogen reserves. Depletion of glycogen reserves has been observed in liver cells exposed to microcystin-LR. Microcystin-LR affects other processes governed by reversible phosphorylation, like muscle contraction, cell division, metabolism, and memory analogously to glycogen metabolism. At extremely high concentrations, microcystin-LR will also inhibit PP2B but does not interfere with PP2C functions (MacKintosh et al. 1990). Therefore only cellular processes that are controlled by reversible phosphorylation involving PP1 and PP2A will be affected by microcystin-LR.

2.2.2 Fate of Microcystin-LR In Animals

2.2.2.1 Exposure To Microcystin-LR

The most common route of animal exposure to microcystin-LR is through oral consumption of water carrying algae containing the toxin within the cells or water containing dissolved toxin. Microcystin-LR is found primarily within the cyanobacterial cells but may be released into the water when the cyanobacteria die (Fitch et al. 1934). Microcystin-LR is very stable in water, resistant to pH extremes, and heat up to 300 ^{o}C (Wannemacher 1989). Freshwater mussels (Eriksson et al. 1989, Falconer and Choice 1992), and fish (Radbergh et al. 1991) are capable of accumulating microcystin-

LR. Consequently consumption of tainted organisms could result in exposure to larger quantities of microcystin-LR than might be found in drinking water. There have been no studies on microcystin-LR absorption through the skin, however exposure to microcystin-LR through skin contact is unlikely as microcystin-LR does not readily cross cell membranes (Eriksson et al. 1990). Microcystin-LR is very water soluble and nonvolatile, therefore inhalation and absorption through the lungs is unlikely, unless microcystin-LR is carried as an aerosol in water.

2.2.2.2 Absorption and Distribution

Microcystin-LR is absorbed from the gut primarily by a single transport mechanism, the multispecific bile acid transporter. The transporter is found in the intestinal, liver and kidney cells. Bile acids aid in absorption of food from the small intestine and Figure 2.4 illustrates how the transporter functions in the absorption process (Frimmer and Zeigler 1988, Alberts et al. 1989). Bile acids are made in the liver and stored in the gall bladder. They are excreted into the small intestine to alkalize the intestinal contents and aid in absorption of fats into the blood. The bile acids are absorbed into the liver from the blood, and then excreted back into the small intestine via the bile duct. This cycling of the bile acids is referred to as enterohepatic cycling and facilitates continual reuse of the bile acids. The transporter is referred to as multispecific because of the wide variety of molecules transported. Transport of microcystin-LR appears to be by the bile acid transporter in both intestinal (Dahlem et al. 1989, Falconer et al. 1992) and liver cells (Eriksson et al. 1990, Hooser et al. 1991b, Runnegar et al. 1981, Runnegar et al. 1991) (Figure 2.4).

The liver has been shown to be the prime target accumulating roughly 50-70% of the total microcystin-LR dose when delivered to experimental animals (Robinson et al. 1989, Meriluoto et al. 1990, Robinson et al. 1991a). The intestine and kidney are the only other organs that have been observed to accumulate significant amounts of microcystin-LR. Other organs have been found to absorb minor quantities of microcystin-LR (Table 2.2). The transport mechanism for microcystin-LR in the kidney has not been experimentally determined . However, the kidney has been shown to have a bile acid transporter similar to the transporter of the intestinal cells in rats, but the transporter was different than the liver bile acid transporter (Burckhardt et al. 1987).

2.2.2.3 Excretion

Only one study has reported findings on the excretion of microcystin-LR, where both liver and kidney excretion was observed (Robinson et al. 1991a). Microcystin-LR was excreted quite quickly by both routes, 75% of the total amount excreted occurred within 12 hours. In total, 23% of the administered dose was excreted after six days, 15% in the feces, and 9% in the urine. The mechanisms of excretion into either the feces or the urine have not been elucidated. In the liver, excretion by a transporter is possible (Frimmer and Zeigler 1988), and therefore the transporter may become saturated resulting in accumulation in the organ. However, microcystin-LR excreted in the feces may not have been absorbed into the body. In the kidney, there are two possible mechanisms of excretion; filtration of blood in the glomeruli and transport from the blood into the tubules by proximal tubule cells (Figure 2.4) (Casarett and Doull 1991). Saturation of the proximal tubule cells may result in accumulation of microcystin-LR.

2.2.2.4 Biotransformation

Biotransformation involves chemically altering the toxin which may decrease its toxicity and improve the body's ability to excrete the toxin. Biotransformation usually involves attaching a molecule to the toxin that increases its solubility in water. This aids in excretion by decreasing the ability of the toxin to passively diffuse back into the blood once excreted into the urine or feces. The majority of the biotransformation enzymes are located in the liver but some enzymes are present in other organs as well (Casarett and Doull 1991). Biotransformation of microcystin-LR has been observed to occur in the cytosol of the liver (Pace et al. 1991, Robinson et al. 1991b) and biotransformed microcystin-LR molecules have also been observed in the feces and urine (Robinson et al. 1991a). The identity of the biotransformation products of microcystin-LR have not been determined, but they have been observed to be more water soluble.

2.2.2.5 Summary

The most likely route of exposure to microcystin-LR is through oral ingestion. Microcystin-LR appears to be taken up by the bile-acid transporter in intestinal and liver cells. The majority of microcystin-LR absorbed accumulates in the liver with minor accumulation in intestinal and kidney cells. Microcystin-LR has been observed to be excreted in both the feces and urine. Biotransformation of microcystin-LR has been observed, and biotransformation products have been isolated in the cytosol of the liver, the feces and urine.

2.2.3 Cellular Interactions

2.2.3.1 Microcystin-LR Protein Binding and Hyperphosphorylation

The majority of microcystin-LR absorbed in the liver has been found to be tightly bound to cytosolic proteins (Hooser et al. 1991b, Pace et al. 1991, Robinson et al. 1991a, Robinson et al. 1991b, Yoshizawa et al. 1990). The particular proteins have not been identified but the molecular weight of the protein - microcystin-LR complex has been found to be similar to the additive weight of microcystin-LR and PP1 or PP2A (Robinson et al. 1991b). The microcystin-LR binding proteins were also observed to dephosphorylate a known substrate of PP1 and PP2A (Yoshizawa et al. 1990). The ability of the proteins to dephosphorylate the substrate was inhibited by microcystin-LR indicating that the proteins are most probably PP1 and PP2A. Consistent with these observations, a significant amount of phosphorylated proteins have also been observed in liver cells after administration of microcystin-LR (Eriksson et al. 1990b, Yoshizawa et al. 1990). Among the proteins found to be hyperphosphorylated are proteins for glycogen metabolism, and some of the proteins of the cytoskeleton (Eriksson et al. 1990b).

2.2.3.2 Cytoskeletal Reorganization

The predominant adverse effect caused by microcystin-LR, is reorganization of the cytoskeleton of cells. The cytoskelton consists of many proteins, actin, myosin, intermediate filaments and tubulin filaments (Alberts et al. 1989). These proteins play different roles in the functioning of the cytoskeleton. A major function of the cytoskeleton is acting as the cell framework, providing physical support for the cell. The cytoskeletal proteins that function primarily in the structural role are called intermediate filaments (Lane and Alexander, 1990). The intermediate filaments link cells together providing integrity to organs as a whole (Figure 2.5). However, the cytoskeleton is not a rigid structure, and the structural formation continually changes. During cell division, major cytoskeletal reorganization occurs (Chou et al. 1991, Lane et al. 1982). Intermediate filaments have been found to be naturally phosphorylated during cell division and it is believed that the reorganization of the intermediate filaments is triggered or signalled by reversible phosphorylation (Chou et al. 1989). Liver cells have been observed to lose cell - to - cell adhesion after treatment with microcystin-LR (Hooser et al. 1991a, Muira et al. 1989, Radbergh et al. 1991) and this could be caused by intermediate filament reorganization at the junctions between cells (Lane and Alexander, 1990). Microcystin-LR has been observed to cause hyperphosphorylation of intermediate filaments (Ohta et al. 1992). The reorganization of intermediate filaments has been observed to be similar to that shown in Figure 2.5 (Falconer and Yeung, 1992).

Actin is the most predominant protein of the cytoskeleton (Alberts et al. 1989). Actin is primarily found in a mesh-like cortex just below the surface of the plasma membrane (Figure 2.5). The actin cortex gives mechanical strength to the surface of the cell and also enables the cell to change shape. Myosin is also present in the cortex and it is the interaction of actin with myosin that generate the forces controlling cell shape and movement. The interaction of actin and myosin is controlled by reversible phosphorylation. The specific details of the mechanism are unknown but PP1 has been observed to dephosphorylate myosin (Fernandez et al. 1990). PP1 is possibly associated with the cytoskeleton by virtue of a myosin targeting subunit (Chisholm and Cohen 1988). The PP1/myosin targeting subunit is analogous to the glycogen binding subunit in glycogen metabolism. Inhibition of PP1 by microcystin-LR could then cause phosphorylation of myosin and cause actin reorganization. Actin is also involved in the structure of cellular microvilli. Microvilli are perpendicular projections out of the cell, and act to increase the surface area of the cell (Figure 2.5). Microcystin-LR has been observed to cause the reorganization of actin (Eriksson et al. 1989b, Hooser et al. 1991a). Actin has been observed to move away from the cell membrane, and concentrate at a single location in the cell (Figure 2.5) (Eriksson et al. 1989b, Hooser et al. 1991a, Runnegar and Falconer 1986). The cells have been observed to develop plasma membrane blebs (observed as a blister on the membrane Figure 2.5) at the same time as actin reorganization (Hooser et al. 1991a). Cells have also been observed to become round, develop membrane invaginations, and lose their microvilli.

2.2.3.3 Summary

Microcystin-LR has been found to bind tightly to proteins located in the cytoplasm and associated with membranes. These proteins are believed to be PP1 and PP2A by virtue of the phosphatase activity of the isolated proteins which is inhibited by microcystin-LR, and the finding that the molecular weights of the proteins are similar to PP1 and PP2A. The major effect of microcystin-LR on the liver cells is reorganization of the cytoskeleton. The reorganization of the cytoskelton results in plasma membrane blebbing, and loss of cell - to - cell adhesion. Reorganization of the cytoskeleton by inhibition of protein phosphatases, is believed to be the cellular mechanism underlying microcystin-LR toxicity.

2.2.4 Systemic Effects

2.2.4.1 Liver Effects

The liver has basically two cell types, parenchymal cells which perform most of the liver functions, and sinusoidal cells that line the blood capillaries that run through the liver acting as a porous membrane (Figure 2.4) (Alberts et al. 1989). Damage to the liver by microcystin-LR initially involves the functional cells (Hooser et al. 1991a). The first effect observed is plasma membrane blebbing because of the cytoskeletal rearrangement caused by microcystin-LR. No other hepatocyte degenerative changes are seen until long after plasma membrane shape changes have been observed. With the loss of cell - to - cell adhesion, the liver architecture is disrupted and hepatic lesions develop consisting of a widening of the spaces between functional cells with occasional invaginations in the plasma membrane (Hooser et al. 1990, Muira et al. 1989, Radbergh et al. 1991). Consequently, blood leaks into the liver, filling the spaces created by the loss of cell - to - cell adhesion. This causes the livers to significantly enlarge, increase in weight and become dark red. In some cells, the plasma membrane breaks causing leakage of liver enzymes and liver cellular debris into the blood. Whole liver cells have also been observed in the blood and other tissues as a result of the loss of the organ architecture. Destruction of the parenchymal and sinusoidal cells causes lethal intrahepatic haemorrhage and/or hepatic insufficiency.

2.2.4.2 Gastrointestinal Effects

Experiments with isolated intestinal cells have shown that they become deformed in a similar manner to liver cells treated with microcystin-LR (Falconer et al. 1992). Haemorrhages in the intestine have also been observed in experimental animals (Galey et al. 1987, Jackson et al. 1984). However, the effects that have been observed on the intestine are not nearly as severe as those described for the liver. The intestinal cells are exposed to the highest concentrations of microcystin-LR, as all the microcystin-LR that enters the body is presumably transported through the intestinal cells. The intestinal cells have been observed to accumulate only a small amount of microcystin-LR (Robinson et al. 1989, Meriluoto et al. 1990, Robinson et al. 1991a) suggesting that the bulk of the microcystin-LR absorbed is transported to the blood without interacting with the intestinal cell contents.

2.2.4.3 Kidney Effects

There have not been any studies with microcystin-LR specifically on the effects caused to isolated kidney cells or whole organs. Therefore, it is not known if kidney damage is related to direct interaction with microcystin-LR, or a secondary effect sustained after the extensive liver damage. Intact liver cells and small amounts of necrotic liver cellular debris have been observed in the kidney glomeruli and peritubular capillaries after administration of microcystin-LR to mice and rats (Hooser et al. 1989). Lesions and cell damage have been observed in the glomeruli and in the collection tubules (Hooser et al. 1990, Meriluoto et al. 1989, Radbergh et al. 1991). The weight of the kidney has also been observed to increase similar to observations in the liver. Blood concentrations of nitrogen compounds have also been observed to increase at roughly the same time as kidney lesions suggestive of damage to the tubules (Hooser et al. 1989, Muira et al. 1989).

2.2.4.4 Respiratory Effects

Like the kidney, there have been no specific experiments conducted with the lungs. Liver cells and cellular debris have been observed in the lungs after treatment of animals with microcystin-LR (Hooser et al. 1989). The observed effects in experimental animals are respiratory congestion, haemorrhaging, and bronchopneumonia (Hooser et al. 1989, Falconer et al. 1988, Falconer et al. 1981, Slatkin et al. 1983). It is not known if the observed damage is a result of the presence of liver material or is caused by direct interaction of microcystin-LR on respiratory cells.

2.2.4.5 Circulatory Effects

A significant amount of blood infiltrates the liver when the architecture of the organ has been severely altered by microcystin-LR. In the extreme, the loss of blood from circulation results in circulatory collapse (Hooser et al. 1990).

2.2.4.6 Summary

The primary effect of microcystin-LR is on the liver. Microcystin-LR causes destruction of the liver parenchymal cells causing subsequent damage of the sinusoidal cells and collapse of the liver architecture. Blood infiltrates the damaged liver causing liver haemorrhage and circulatory shock which leads to the death of the animal. The effects caused to the intestine, kidney, and respiratory system are not as severe as those in the liver. Consequently they have not been mechanistically characterized.

2.2.5 Carcinogenesis: Initiation, Promotion and Progression

Cell growth or proliferation is a normal process that is continually occurring in cells. Cell proliferation is regulated by a balance in the expression and function of growth activators and tumor suppressors (Goldberg et al. 1991). Abnormal cell proliferation occurs if the balance of growth activators and tumor suppressors is upset. This can occur by an event that inhibits the expression or function of tumor suppressors, or enhances the expression or function of growth activators. Carcinogenesis can be considered a three step process, initiation, promotion and progression (Casarett and Doull 1991). Initiation of carcinogenesis can be thought of as a reaction involving the genetic material (chromosomes or DNA) of the cell that results in an heritable change to the genetic material. Initiation has been observed to be irreversible in rat liver cancer experiments (Pitot and Campbell, 1988). A chemical that is capable of causing such a reaction is called an initiator. Tumor promoters do not interact primarily with the DNA, but enhance abnormal growth of a cell. The effect of the tumor promoter is to cause the cell to begin proliferating, i.e. cell division. In cells that have been initiated, this cell proliferation results in the growth of many small benign tumors. Tumor promotion has been observed to be a reversible process in rat liver cancer experiments (Pitot and Campbell 1988). Removal of the tumor promotion stimulus has resulted in the disappearance of the promoted cell populations. The progression stage of cancer has not been fully defined but this stage is characterized by irreversible benign and/or malignant tumors (Pitot and Campbell 1988).

2.2.5.1 Microcystin-LR as a Tumor Initiator

There are several tests which determine the ability of a chemical to cause changes in the DNA. There have been no reported studies indicating microcystin-LR acts as an initiator. However, studies with three purified cyanobacteria extracts, where the toxins were not positively identified, produced a significant amount of chromosome (DNA) breakage. Chromosome damage with the cyanobacteria toxins was more closely dose-related than breakage observed with benzene. Benzene, and sodium arsenite produced much lower chromosome damage than did the cyanobactrial toxins and PCB (3,4,3',4') produced damage that was similar (Repavich et al. 1990).

2.2.5.2 Microcystin-LR as a Tumor Promoter

Tumor promotion capabilities are determined with a two stage experiment. First, a known initiator is applied to the experimental animal to induce DNA damge, and then the potential tumor promoter is given to determine if it will induce abnormal growth resulting in a tumour. Microcystin-LR has been shown to be a powerful tumor promoter with the two stage experiment (Nishiwaki-Matsushima et al. 1992). Rats were given a liver specific initiator (diethylnitrosamine, DEN) and then fed microcystin-LR at various doses (10, 25, and 50 μg/kg twice per week). They observed a dose dependent increase in the number and size of focal lesions in the livers of the rats fed microcystin-LR.

The doses used in these experiments are much higher than would be encountered by consumption of drinking water, but the experiments determine the potential for tumor promotion by microcystin-LR . In comparison with other tumor promoters, microcystin-LR is one of the strongest tumor promoters known. The tumor promoting activities of phenobarbitol, hexachlorocyclohexane, cyprotone acetate, and chenodeoxycholic acid have been reported in the μg/kg/day range (Nishiwaki-Matsushima et al.1992). The tumor promoting activities of microcystin-LR are observed in the μg/kg/day range whereas dioxin, the most powerful tumor promoter known, promotes when administered in the ng/kg/day range (Dragon et al.1992, Tritscher et al.1992).

Inhibition of protein phosphatases has been presented as a general mechanism of tumor promotion (Fujiki 1992). Inhibition of protein phosphatases results in the hyperphosphorylation of some tumor suppressor proteins. Hyperphosphorylation may inactivate the suppressor proteins and this would cause an imbalance between growth activators and tumor suppressors in the cell. Microcystin-LR may be acting as a tumor promoter by causing the hyperphosphorylation of tumor suppressors.

2.2.6 Case Reports and Epidemiology of Microcystin-LR

2.2.6.1 Case Reports of Animal Poisoning

Animal deaths from consumption of cyanobacterial waters have been reported from most parts of the world. An early report of animal intoxication from cyanobacteria, by G. Francis in 1878, detailed the symptoms of death observed in sheep, horses, dogs, and pigs after consuming lake water containing cyanobacteria. There are several other early reports of animal deaths (including fish and birds) from consumption of cyanobacteria waters and a summary of American reports up to 1934 was presented by Fitch and coworkers (1934). Steyn (1945) reported that many thousands of stock, mainly sheep and cattle, had died from cyanobacteria poisoning during the previous 25 to 30 years in the north-eastern Free state and south-eastern Transvaal, Africa. They observed that the cyanobacteria contained a potent liver toxin that was released into the water from the cyanobacteria when they died. In the summer of 1959, several dogs, geese, fish, cattle, and horses died in Saskatchewan after consumption of water containing cyanobacteria (Dillenberg and Dehnel 1959). Autopsy of two dogs revealed swollen and spotty liver, excess fluid in the lungs, and inflamed intestines. Sheep consuming water containing cyanobacteria developed lesions in the liver (Main et al. 1977). In the mildest cases, the livers were swollen with a scatter of dead cells present but in the severe cases virtually all the liver cells were destroyed. The bile duct was also observed to be swollen. Some of the sheep also developed pneumonia. Twenty dairy cows consuming water containing cyanobacteria developed similar symptoms of cyanobacteria intoxication as those described for the sheep (Galey et al. 1977). However, eleven of the cows survived, and one week after the onset of the symptoms the cows seemed fine except that they had an elevation of some liver enzymes suggestive of liver damage. In 1989, 20 sheep, and 15 dogs were reported to be killed from exposure to water with an cyanobacteria bloom containing microcystin-LR (Lawton and Codd 1991).

Most incidents of animal intoxication have occurred when the cyanobacteria bloom has been blown near to the shore where the animals consume water containing intact cells. When animals become intoxicated the symptoms generally are stupor and unconsciousness, falling and remaining quiet, vomiting, convulsive movements or twitching, diarrhoea, and loss of appetite or desire for drink.

2.2.7.2 Case Reports of Human Poisoning

There have been reports of human contact and ingestion of waters containing cyanobacteria but there have been no reports of human death. Most of the incidents are characterised by circumstantial evidence described in retrospect. A number of historical incidents, dating back to 1887, of human sickness in Australia probably caused by microcystin toxins have been summarized by Hayman (1992). The incidents are related to a once prevalent sickness in outback northern and central Australia, known variously as "Barcoo fever, Barcoo spews, Barcoo sickness", characterised by symptoms that are very similar to those described for the microcystin toxins.

Several episodes of human illness occurred in Saskatchewan in the summer of 1959 after contact with water containing cyanobacteria blooms (Dillenberg and Dehnel 1960). Ten children were sick with diarrhoea and vomiting after bathing in water from a lake covered with cyanobacteria. *Anabaena* (which have been reported to produce microcystin-LR, Harada et al. 1991) cells were found in the stool of one child. One man developed signs of gastroenteritis, head-ache and nausea after swimming waters containing cyanobacteria. Another man reported to have swallowed half a pint of these waters, developed nausea, painful diarrhoea and vomited several hours later. *Microcystis* cells were found in his feces.

Several reports of human illness associated with water containing cyanobacteria blooms were reported in 1981 (Billings 1981). One incident involved twelve children and one adult having contact with a bloom of cyanobacterium *Anabaena* in Arrowhead Lake, Monroe County, Penn.. They showed symptoms ranging from gastrointestinal involvement to hayfever within a maximum of 12 hours. The symptoms lasted a varying amount of time but none longer than 5 days.

In Staffordshire, England, 1989, eight soldiers reported influenza - like symptoms after swimming and canoeing in water containing a cyanobacteria bloom. Microcystin-LR was identified in the bloom material (Lawton and Codd 1991). Two men became seriously ill and had to report to hospital (Turner et al. 1990). Their symptoms included sore throat, blistering in and around the mouth, abdominal pain, vomiting, diarrhoea, fever and pleuritic pain on the left side. They also developed signs of an atypical pneumonia which persisted for about four days. One man had elevated liver enzyme levels in the blood stream indicative of liver damage.

There have been several incidents where populations have become ill after consuming drinking water tainted with cyanobacteria. In 1930, between 8,000 and 10,000 people in Charleston, W.Va. suffered from an acute intestinal ailment (Tisdale 1930). The illness was characterized by a sudden onset, pain in the region of the stomach, usually nausea or vomiting or both, and followed by diarrhoea of varying severity. The water supply had a bloom of cyanobacteria which was treated with copper sulphate and powdered activated carbon in addition to conventional treatments. The bacteriological records did not indicate any pathogens in the water. However neither pathogenic protozoa or viruses would have been detected at this time and they may have caused the observed illness. Other incidents along the same river also occurred but were not as well characterized (Veldee 1931).

In Salisbury, Rhodesia (now Zimbabwe), gastroenteritis was observed in children consuming water from a source which had cyanobacterial blooms (Zilberg 1966). The illness was characterized primarily by vomiting and diarrhoea, some children also suffered convulsion and fever, and over-breathing of acidosis. The outbreak occurred annually in winter, from 1960-1965, at the same time as the presence of large cyanobacteria blooms in the water supply. The cyanobacterial blooms were predominately *Microcystis* and their abundance coincided with the incidence of gastroenteritis (Marshall 1991). Children not using the water supply did not suffer from the illness.

A similar gastrointestinal incident involving 138 children and 10 adults occurred on Palm Island, Australia in 1979, after consuming water that had a large cyanobacterial bloom (Byth 1980). The cyanobacterial bloom was treated with copper sulphate (Bourke et al. 1983). The illness was characterized by vomiting, and enlargement of the liver (Byth 1980). Over the first few days, 82% of the children developed acidosis and abnormally low potassium concentrations in the blood. After the acidosis shock, 24% of the children developed profuse diarrhoea.

In the town of Armidale, Australia, 1983, a cyanobacterial bloom of *Microcystis aeruginosa* occurred in the drinking water reservoir, and the bloom was treated with copper sulphate (Falconer et al. 1983a). In residents consuming the water, a significant increase in the blood level of the liver enzyme gamma-glutamyl-transpeptidase occurred indicative of liver damage.

There is only one situation where the tumor promoting capabilities of the cyanobacteria toxins has been considered. In China, the incidence of liver cancer has been related to the source of drinking water used (Delong 1979, Shun-Zhang 1989). In areas of Quidong County, Jiangsu province, showing high rates of liver cancer, the drinking water supply was mainly rainwater collected in ditches between houses and fields. The water of the ponds and ditches of Quidong county has been reported to be contaminated with high levels of cyanobacteria (Nishiwaki-Matsushima et al. 1992). In the low liver cancer incidence areas, the water supply is from flowing rivers or wells, with only a few ditches being used for drinking water. In four surveys conducted, there were 79 cases per 100,000 of liver cancer among ditch water drinkers, and 1.3 cases per 100,000 among well water drinkers.

2.2.6.3 Summary

Death and illness from cyanobacteria has been documented in the literature for 115 years. Death to animals usually occurs when the bloom material has been blown to the shore from which the animals drink causing an exposure to a much greater concentration of the toxin. The symptoms described from accidental intoxication are very similar to that observed with laboratory animals. No human deaths have resulted from intoxication but very many people have been severly ill. This implies that human beings are not immune to the cyanobacteria toxins and exposure to a high enough concentration could be fatal. From the case reports that have been documented, most incidents of population exposure have resulted after treating the toxic bloom with copper sulphate.

2.3. ANALYTICAL TECHNIQUES AVAILABLE

2.3.1 Mouse Bioassay

The mouse bioassay basically consists of finding the minimum amount of toxin required to kill a mouse, and comparing this value with lethal doses of a known amount of toxin. The mouse bioassay is an old quantitation technique. It was used to follow the removal capabilities of several water treatment applications in 1942, (Wheeler et al. 1942), and a detailed description of the technique was presented by Hughes et al. (1958). The test had two advantages in that it was inexpensive, and results were obtained within a few hours (Carmichael 1992). A major disadvantage with the test is that it does not detect toxins at low concentrations, especially in finished drinking waters. Therefore the test is not that useful for monitoring drinking water for public safety. Another major concern is that the test involves killing many experimental animals.

2.3.2 High Performance Liquid Chromatography/Ultraviolet Light Detection

HPLC/UV detection is a useful technique for screening water samples for microcytins. Sample injection can be automated for processing large numbers of samples. The important advantage of HPLC is that it can distinguish between microcystin analogues. However, HPLC/UV detection, although more sensitive than the mouse bioassay, is not sensitive enough to detect microcystin in the water at concentrations less than 1 μg/L. Another disadvantage is that HPLC requires initial sample preparation and clean-up (Kenefick 1991). The technique has not been developed into a standard method easily used by monitoring agencies or testing laboratories (Carmichael 1992).

2.3.3 Protein Phosphatase Bioassay

The protein phosphatase bioassay (Holmes 1991) is currently being developed for quantitation of microcystin-LR in water samples. Quantitation is determined by following the inhibition of protein phosphatases PP1 and PP2A. The technique is extremely sensitive and is capable of detecting microcystin-LR in finished and raw water samples at concentrations as low as 50 ng/L. The assay is quite fast, and samples can be quantified in a few hours. Another advantage is that only small sample volumes are required and the technique does not require sample preparation or clean-up. The assay does not distinguish between microcystin analogues, and measures the inhibition activity of the water as a whole. The major disadvantage of the technique is that the assay requires substantial preparation of reagents and the use of radioactive material. Isolation of the protein phosphatases is also difficult but they may soon be commercially available. These factors will limit the ability of the assay to become a routine standard test at water treatment facilities that have cyanobacteria bloom problems.

2.3.4 Immunoassay Techniques

There has been some research into immunoassay techniques for detection of microcystin toxins, summarized by Carmichael (1992). The technique was capable of detecting microcystin toxin in drinking water and animal tissue.

2.4 FATE OF MICROCYSTIN-LR IN DRINKING WATER TREATMENT

2.4.1 Pretreatment of the Raw Water Source

Treatment strategies differ in effectiveness with respect to both the type of algae and cyanobacteria, and the type of problem to be solved. Cyanobacteria in drinking water sources are a concern because they produce a variety of toxins, and several potent taste and odour compounds. However, pretreatment should be aimed at arresting both cyanobacteria and green algae growth because they both produce a large amount of organics, primarily carbohydrates, amino acids and protein. There is some concern with amino acids and proteins since it has been established that reactions of chlorine and chloramine with amino acids can produce odourous compounds (Hrudey et al. 1988), and chlorinated organics (Peters et al. 1992, Wardlaw et al. 1991). Oliver (1983) compared the amount and type of chlorinated organics produced from fulvic acids, cyanobacteria and green algae. Trihalomethanes (THM), dihaloacetonitrile (DHAN), haloacetic acids (HAA), haloketones, chlorophenols, chloropicirin, chloral hydrate, and cyanogen chloride have been observed as a result of chlorination (Krasner et al. 1989). Pretreatment strategies should be orientated towards preventing bloom formation as the total quantity of toxin in the bloom increases with growth. Pretreatment strategies consist primarily of chemical application to the water as biological and physical treatments have not been fully employed (AWWA 1987).

2.4.1.1 Chemical Treatment

Copper Sulphate

Copper sulphate is the most widely used pre-treatment chemical used for control of algal blooms in north america (AWWA 1987). Toxicity of copper sulphate to algae has been attributed to the concentration of free copper ion (McKnight et al. 1983). Complexation of free copper ion with organic matter and carbonate species appears to decrease the toxicity of the copper ion (Horne and Goldman 1974, Andrews et al. 1977). Above neutral pH copper carbonate complexes are predicted to be the major copper species and organic complexes will only be significant in waters with very high organic matter (McKnight et al. 1983). The implication for copper algal control strategies is that there is a better chance that the treatment will be effective at low pH, especially within the pH range of 6 to 8 (Elder and Horne, 1978). Detailed copper speciation diagrams are available in the literature (Elder and Horne 1978, McKnight et al. 1983, and AWWA 1987).

At low copper concentrations, 5 to 10 μg/L Cu, nitrogen fixation by cyanobacteria is reduced (Horne and Goldman 1974), with shifts in community structure resulting in near elimination of cyanobacteria and heavy predominance of green alga and diatoms (Elder and Horne, 1978). Typical blooms of copper tolerant green algae occur in dugouts with a history of copper treatments (Peterson and Swanson 1988). The green algae blooms flourish because the copper also kills zooplankton, that graze on green algae.

Copper sulphate is usually applied at much greater concentrations, approximately 100 μg/L or more, to kill algal blooms (McKnight et al. 1983).

The treatment is effective in the temporary killing of algae and cyanobacteria, but the negative results are rapid dissolved oxygen depletion by decomposition of the released cytoplasm from dead algae, accelerated phosphorous recycling from the lake bed and recovery of the algal populations within seven to twenty-one days (Hanson and Stefan 1984). Occasional fish kills attributed to oxygen depletion or copper toxicity have occurred, and the disappearance of macrophytes, and reductions in benthic macrovertebrates has been observed (Hanson and Stefan 1984). Other long term effects are shown to include copper accumulation in the sediments, tolerance adjustments of certain species of algae to higher copper sulphate dosages, and a shift of species from green algae to cyanobacteria and from game fish to rough fish (Hanson and Stefan 1984, McKnight et al. 1983).

Copper sulphate also causes the release of toxin from the cyanobacterial cells. In 1948, Prescott demonstrated that treatment of cyanobacterial cells with copper sulphate can cause the killing of fish by other means than depletion of dissolved oxygen. In a batch experiment, cyanobacterial cells analyzed by scanning electron microscope after treatment with 0.64 mg/L copper sulphate, were found to be lysed, and microcystin-LR was substantially released from the cyanobacteria into the water (Kenefick et al. 1993). The release of toxin into the raw drinking water after copper sulphate treatment of cyanobacteria blooms may have been a factor in at least three reported incidents of gastrointestinal illness (Tisdale 1931, Bourke et al. 1983, and Falconer et al. 1983a), and a massive fish kill (Sawyer et al. 1968).

Where high and relatively stable nitrate levels support a rather diverse phytoplankton community, and where nitrogen fixation is not a major source of nitrogen input, the system is likely to be relatively insensitive to copper treatment (Elder and Horne, 1978). Where nitrogen fixation is a major component of the nitrogen budget, copper treatment can be effective at concentrations 1/200 of those used to kill blooms to inhibit nitrogen fixation (Horne and Goldman 1974). Algal and cyanobacterial control programs involving use of copper should be planned for maintenance of a manageable and ecologically beneficial algal and cyanobacterial communities and not for drastic reductions in the standing crop (Elder and Horne, 1978). Because copper sulphate treatment of toxic cyanobacterial blooms in drinking water sources will result in the rapid release of toxin into the water, treatment of blooms must be carefully considered and the treated waste should not be consumed for several weeks after treatment (Kenefick et al. 1993).

Lime Treatment

An alternative to copper sulphate is the application of lime (calcium hydroxide). The principal function of lime is to precipitate phosphorus and remove it from the water column (Prepas et al. 1990). Lime added to a hypereutrophic lake in Alberta caused a decrease of 33% of phosphorus from the previous summer (246 to 75 µg/L) possibly by enhanced apatite formation because of increased Ca in the sediment (Prepas et al. 1990). A short term decline in total phosphorous was not observed. High doses of lime, 250 mg/L, removed both algae and phosphorus from the water column but low doses 17 mg/L were ineffective. The algae disappearance may be caused by lack of phosphorus, coprecipitation with calcium, and/or an inability to grow at high pH, the pH increased to 11.0 (Peterson 1989). An advantage of lime treatment is that it does not appear to cause the release of toxin from the algae cells that has been observed with copper sulphate (Kenefick et al. 1993). In a batch experiment, lime added at 100 mg/L, precipitated cyanobacteria cells without causing excessive cell lysis resulting in the release of microcystin-LR to the water.

Detrimental effects on the aquatic environment from lime treatment have not been thoroughly investigated. Prepas et al. (1990) reported no detectable negative impact on invertebrates and vertebrates in the lake, but the macrophyte species shifted from rooted aquatic plants to a non-rooted variety. However, this effect may be of importantance, when the areal coverage of submersed macrophytes was reduced significantly by grazing of grass carp, the result was increased nutrient concentrations and abundance of all major phytoplankton divisions. Summer cyanobacteria density and relative abundance to the phytoplankton increased over time coincident with macrophyte elimination (Macenia et al. 1992). The effect of increase in pH after lime treatment has not been addressed.

2.4.1.2 Biological Control

Biological control of algal and cyanobacteria blooms has not been extensively developed. For farm dugouts and manageably sized reservoirs the use of duckweed to channel nutrients into a biomass that does not present a problem has met with some success (Peterson 1989). Duckweed ponds should be aerated and supplied with nitrogen as most dugouts are low in nitrogen in the summer months. The duckweed should be harvested so they do not pose further organic loading to the raw water when they decompose. If properly managed, duckweed will remove large quantities of phosphorus and severely restrict sub surface phytoplankton growth through a combination of phosphorus uptake and physical shading. The method was successful in both corral and whole dugout experiments.

2.4.1.3 Physical Treatment

For small reservoirs, covering the reservoir is effective but aeration is also required to keep oxygen levels (Peterson 1989). Aeration to destratify the reservoir has met with some success when utilized as one phase of an overall algae control program (AWWA 1987). Removal of nutrients entering the reservoir, particularly phosphorous, by coagulation, sedimentation and filtration can be an effective means of controlling cyanobacteria blooms (Bernhardt 1983, Peterson 1989, Prepas et al. 1990). Phosphate precipitation can be achieved by precipitation with lime (Peterson 1989, Prepas et al. 1990) or ferric salts (Bernhardt 1983). Removal of nutrients from the reservoir influent can prevent eutrophication of the reservoir pool and may minimize cyanobacteria blooms.

2.4.2 The Removal of Cyanobacterial Toxin with Treatment Processes

The fundamental treatment concepts for water containing cyanobacterial toxin were developed in the first half of the century from incidental and experimental intoxication episodes. The specific details of the experiments are given in Tables 2.3 and 2.4.

Wheeler et al. (1942) employed a mouse bioassay to examine the toxicity removal capabilities of alum coagulation, chlorination, and activated carbon from an extract made from *Microcystis aeruginosa*. Neither alum coagulation or chlorination was able to decrease the toxicity of the extract.

When activated carbon was added in large amounts and the suspension filtered, no toxicity could be demonstrated with doses 4 times the concentration of the pretreatment lethal dose. However carbon added in small amounts, corresponding to those used in water treatment practice, resulted in a 50% reduction in the toxicity. The carbon residue was also toxic for mice, the toxic action being delayed (10 hours). Stewart et al.(1950) and Shelubsky (1951) also reported that activated carbon removed the toxic principle of *Microcystis aeruginosa* from water and that efforts to elute the toxin from the carbon were unsuccessful. Unfortunately the details of these early experiments were not described in full, but the conclusions drawn from the experiments are in agreement with contemporary results.

Hoffman (1976) conducted laboratory scale water treatment experiments with 5 mg of purified toxin isolated from *Microcystis* dissolved in 500 ml of test water. The results indicated that powdered activated carbon (PAC), 100 mg/L, or a granular activated carbon (GAC) column, were effective in removing the toxins below the lethal concentration in a mouse bioassay. Conventional unit processes, $FeCl_3$ coagulation-flocculation, sedimentation, filtration, disinfection (pre and post-chlorination), and sand filtration were ineffective at removing the toxic fractions.

A pilot plant analysis of activated carbon and rapid sand filtration treatment demonstrated that activated carbon was effective at removing toxin when applied as a 7-8 cm. layer to the top of sand filtration bed (Falconer et al., 1983b, Falconer et al. 1989). A mouse bioassay was employed for determining toxin removal in these experiments. Activated carbon that had been in use for approximately one year was also effective at removing toxin suggesting that adsorbed natural organic matter does not inhibit adsorption of the toxin. The sand filtration bed without activated carbon was not effective at removing the toxicity. GAC column tests were also conducted with similar results. Several brands of carbon were compared in both the column test and the pilot plant study. The effectiveness of the different brands of carbon varied somewhat in both the column and pilot plant experiments but no particular carbon type was substantially better than any other. Efforts to elute the toxin from the carbon were unsuccessful. Several brands of PAC were also examined in the laboratory with 0.1 to 2.0 g PAC added per 100 mL. Residual toxicity was only found in the 0.1 g/100 mL samples and there was not a significant difference in the carbon types. The concentration of toxin in the experiments was not determined but the LD_{100} was 17 mg dry bloom/kg mice. The authors suggested the concentration was 10 to 100 times the concentration of algal toxins likely to be encountered in water treatment practices.

Conventional treatment trains with addition of ozonation, PAC, and GAC were examined in the laboratory to assess toxicity removal from lyophilized cyanobacteria cells of *Microcystis wesenbergii* and *M. virdii* and *Oscillatoria* agardhii (Himberg et al. 1989, Keijola et al. 1988). These experiments employed HPLC/UV detection to follow concentration changes in the experiments. Standard toxin solutions were prepared by isolation of toxin from the bloom material used in the experiments. The hepatotoxicity of the standard toxin was verified by mouse bioassay but the identity of the toxin was not determined. The initial concentration of toxin in the bloom material ranged from 30 to 58 µg/L. The results confirmed that conventional water treatment processes, (coagulation/sedimentation, sand filtration, and chlorination), are not effective in removing algal toxins. Alum and ferric chloride coagulants were tested. The results demonstrated that ozonation is an effective treatment for reducing algal toxicity. The concentration of ozone supplied was a critical parameter with a concentration of 1 mg/L removing the toxin below the detection limit. GAC column filtration was also observed to reduce the toxin concentration below the detection limit. PAC at 5 mg/L, 20 minutes contact time, was not that effective in removing the toxic fractions, removing only 13 to 34% of the toxicity.

Pilot plant experiments with freeze dried and fresh algae were conducted to simulate a bloom after dosing with copper sulphate or when cells have died from old age compared with an untreated fresh bloom (Keijola et al. 1988). The toxin concentrations were determined as discussed in the previous paragraph. The toxin concentration was 15 μg/L freeze dried, and 50 μg/L fresh algae. Preozonation removed 50% of the freeze dried toxin, and 90% of the fresh algae toxin. Ozonation caused flotation of the algal cells in the flocculation basin. PAC applied at 20 mg/L removed 90% of the freeze dried toxin and 99% of the fresh toxin. Dosages of 100 and 200 mg/L removed the toxins below the detection limit. The slightly better removal of the fresh toxin was thought to be caused by better removal of the algal cells with toxin inside. A laboratory scale slow sand filtration apparatus was also effective in removing toxicity from the water (Keijola et al. 1988).

2.4.3 Summary

Water treatment experiments conducted at the laboratory and pilot plant scale have shown that coagulation, filtration and chlorination are not effective treatments for microcystins. Activated carbon filtration and powdered activated carbon have been effective at removing microcystin in both the bench scale and pilot plant experiments. Ozonation has also been shown to remove the toxicity from water samples in pilot plant and lab scale experiments. However, the lack of a sensitive analytical technique has prevented the water treatment experiments to be conducted at practical concentrations of toxin, and assess the degree of removal by the various treatments. The high concentrations used in the experiments may cause an overestimation of carbon adsorption, particularly with respect to the effects of background organic matter (Najm et al. 1991). With the emergence of the protein phosphatase bioassay, evaluation of microcystin concentrations likely to be encountered in drinking water concentrations is now possible. The toxins used in the experiments were not identified. The availability of purified algal toxins, mainly microcystin - LR, will enable future experiments to be conducted with known toxins.

Table 2.1 Serine/Threonine Protein Phosphatases.

Protein Phosphatase	**Binds Inhibitors 1 and 2**	**Requires Calcium ion**	**Requires Magnesium ion**
Type 1			
PP1	YES	NO	NO
Type 2			
PP2A	NO	NO	NO
PP2B	NO	YES	NO
PP2C	NO	NO	YES

Table 2.2 Tissue Distribution of microcystin-LR in Mice

Research Group	**Liver**	**Intestine**	**Kidney**	**Carcass organs**
Robinson et al.1989	50-60%	7%	1%	10%
Meriluoto et al.1990	70%	10%	3-5%	10%
Robinson et al.1991	70%	10%	1-5%	10%

note; amounts are in % of injected dose

Table 2-3 Comparison of Conventional Water Treatment Experiments

STUDY AND TOXIN USED	CONVENTIONAL TREATMENT	OBSERVATION
Wheeler et al.1942 - *Microcystis aeruginosa* extract, - 0.25 mL caused lethality, concentration = 16 µg/L	•Alum • filtered for removal • chlorination, 100 ppm and 8.4 ppm, 12 hr C.T.	• no decrease in toxicity in mouse bioassay at any stage
Hoffman 1976 • *Microcystis aeruginosa* extract • 0.5 mL caused lethality	• 500 mL volume •prechlorination 5mg/L $Ca(OCl)_2$, 30 min. C.T. •$FeCl_3$, 20 mg/L •flash mix 2 min. •8 min. floc. •10 min sed. •sand filtration •chlorination 5mg/L $Ca(OCl)_2$, 30 min. C.T.	• no decrease in toxicity in mouse bioassay at any stage
Himberg et al.1989 •*Microcystis wesenbergii* extract •*Microcystis verdii extract* concentration = 60 µg/L and 40 ug/L •*Oscillatoria agardhii* concentration = 40 µg/L	• 1 L volume • Alum, 36 mg/L, and 71 mg/L, pH=6.3 with lime adjustment • rapid mix, • 30 min. floc. • 2 hr sed. • sand filtration • chlorination, 0.5 mg Cl_2/L of NaOCl, 20 min. C.T.	• increase in coagulant dose had no effect • observed 11 to 30 % reduction in concentration • only the final water was tested
Himberg et al.1989	• 1 L volume •$FeCl_3$, 55 mg/L •everything else same as above	•less removal with $FeCl_3$ than with Alum •0 to 16% reduction in concentration

note: C.T. = contact time

Table 2-4 Activated Carbon and Ozone Removal of Microcystin Toxins.

STUDY AND TOXIN USED	EXPERIMENTAL DETAIL	OBSERVATION
Wheeler et al. 1942 •*Microcystis aeruginosa* extract, • 0.25 mL caused lethality, concentration = 16 μg/L	• activated carbon added at treatment levels • activated carbon added in large amounts	• treatment dose resulted in 50% reduction in toxicity • large dose no toxicity at 4 times lethal dose of control sample
Hoffman 1976 •*Microcystis aeruginosa* extract •0.5 mL caused lethality	• 2 mg of toxin in 250 mL •added 0.1, 0.2,, 2.0, 20, 200, 1000 mg of Pittsburgh Filtrasorb 300	• 800 mg/L dose removed toxin below detection level
Falconer et al. 1983, 1989 •*Microcystis aeruginosa extract* • Lethal dose was 17mg/1kg mouse	• 50 mL of algal extract • added 0.05, 0.125, 0.25, 0.5, 1.0 g carbon • tested several brands	• all brands removed 95% toxicity at 0.5 g •Norit W52, SA5 •Darco HDB, S51, KB •BDH charcoal •Ajax charcoal •May and Baker charcoal were removed 95% toxicity at 0.125 g
Falconer et al. 1983, 1989	• GAC column test •70 g GAC in 3.5 x 18 cm column • flow = 34 mL/min. • 20 L sample passed, 10% solution of extract •analyzed every 5 L	• tested a variety of carbons • Calgon Filtrasorb 100 and 200, Darco Granular, Norit Row 0.8 supra performed the best • breakthrough occurred at 20 L or more

Table 2-4 (continued)

STUDY AND TOXIN USED	EXPERIMENTAL DETAIL	OBSERVATION
Falconer et al.1983, 1989	Pilot plant test • column, 0.6 m diameter, accommodating a 5 m head • a 0.7 m bed of sand • 13 kg of carbon applied on top of sand, the thickness of carbon was 7-8 cm. flow rate = 90 L/min/m^2 • 4 L algal extract with 4000 L	• a variety of carbon samples were tested • breakthrough occurred at 1.2 m^3/kg, for most carbons • Norit ROW 0.8 Supra lasted until 24 m^3 • a sample of pre-loaded carbon had same breakthrough as virgin carbon
Keijola et al.1988 • *Microcystis aeruginosa* •freeze dried material, 15 µg/L • fresh algal material, 50 µg/L	• 100 L pilot plant •CO_2 tower (9.5-13.5 mg CO_2/L) •ALUM, pH adjusted to 6.3 with lime •PAC added as slurry (5 L/h) • at 20 mg/L, 100 mg/L, 200 mg/L into flocculation basin •sedimentation, • filtration (45cm anthracite, 50 cm sand) •Ozone, 1.0-1.5 mg/L • applied after the CO_2 tower	• PAC at 20 mg/L removed 90% of the toxin • 100 and 200 mg/L removed all the toxin • with fresh material ozonation removed 90% of the toxin • with freeze dried, only 50%, the treatment plant reduced toxin from 50 to 10%

Table 2-4 (continued)

STUDY AND TOXIN USED	EXPERIMENTAL DETAIL	OBSERVATION
Himberg et al.1989		
•*Microcystis wesenbergii* extract	• laboratory scale as described previously	
•*Microcystis verdii extract* concentration = 60 µg/L and 40 µg/L	• PAC 5 mg/L, 20 min contact with agitation before flocculation with ALUM/sand filtration	• 13-34 % removal was observed with PAC
•*Oscillatoria agardhii* concentration = 40 µg/L	• GAC column, 12 x 3 cm • flow = 5.1 $m^3/m^2/H$ • after ALUM/sand filtration	• GAC column removed toxin below detection level
	• ozone applied at 1.0 mg/L for 30 min • before ALUM/sand filtration	• ozone removed toxin below detection level

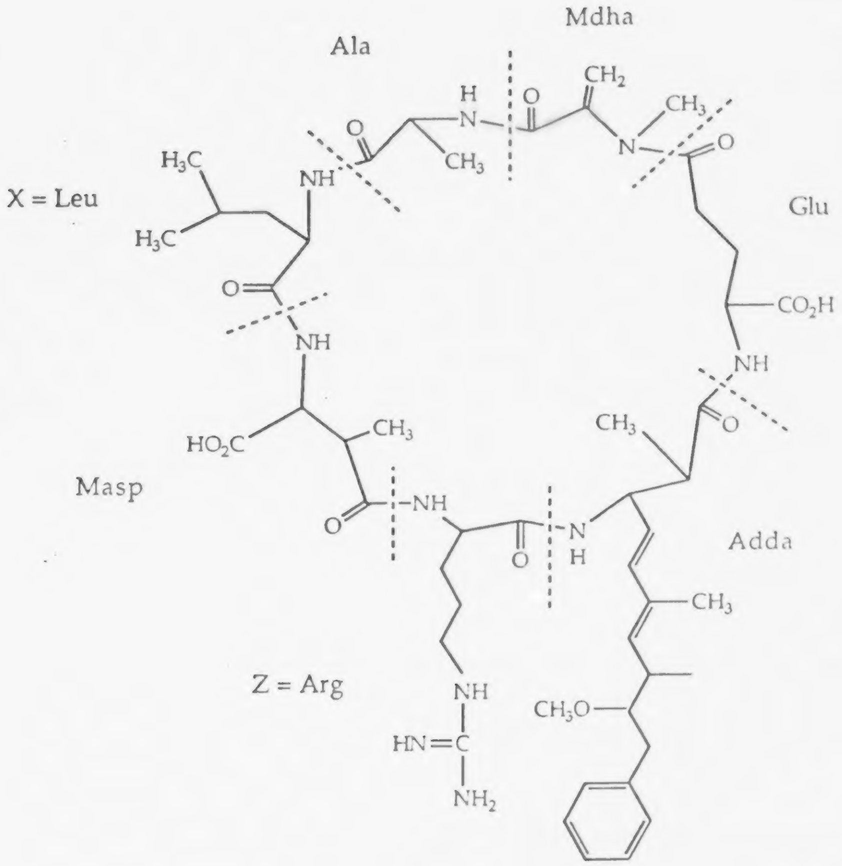

Mdha
Ala
CH$_2$
H
O
N
CH$_3$
O
N
O
H$_3$C
NH
CH$_3$
X = Leu
Glu
H$_3$C
O
CO$_2$H
NH
NH
CH$_3$
HO$_2$C
CH$_3$
O
Masp
NH
N
H
Adda
O
O
CH$_3$
Z = Arg
NH
CH$_3$O
HN=C
NH$_2$

Figure 2.1 Structure of Microcystin-LR.

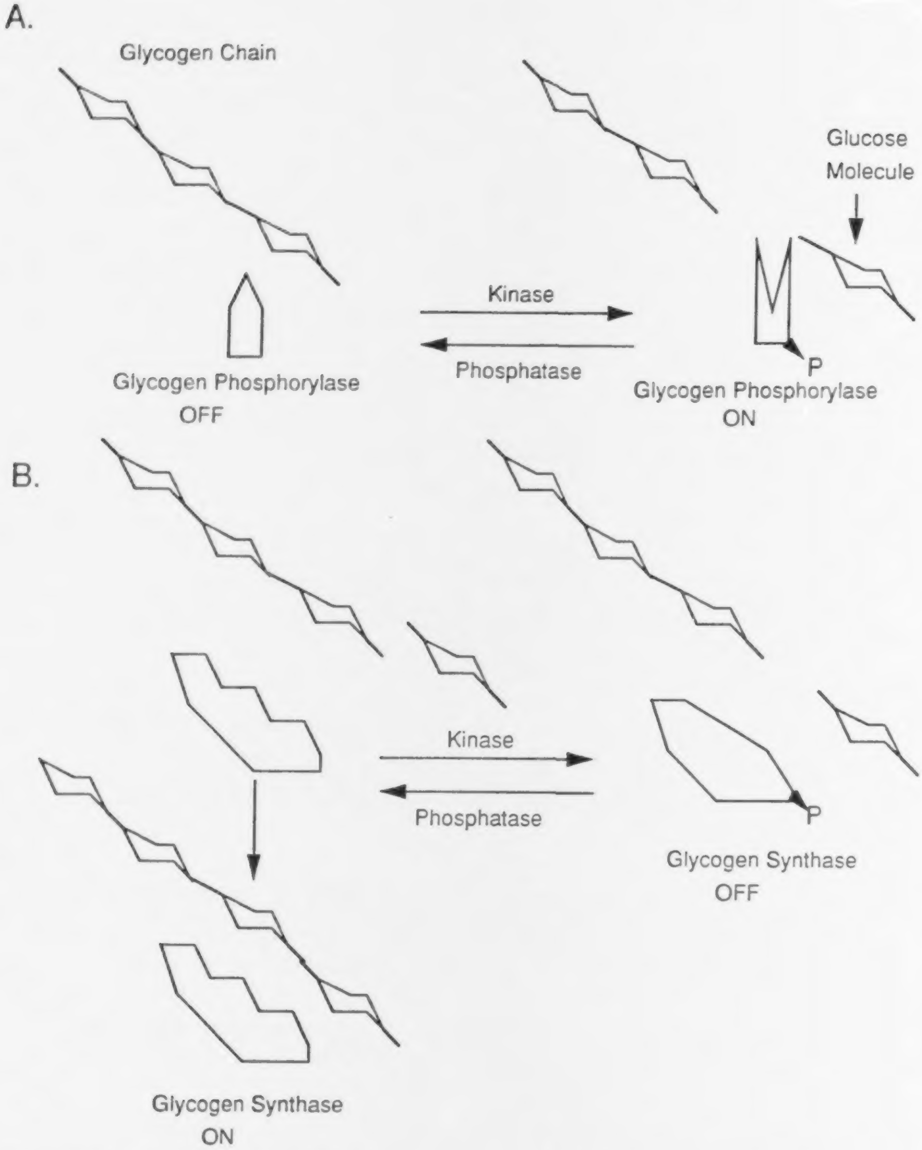
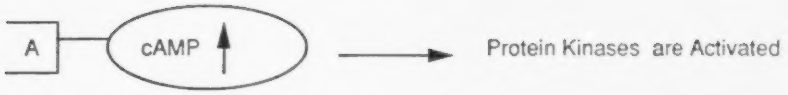
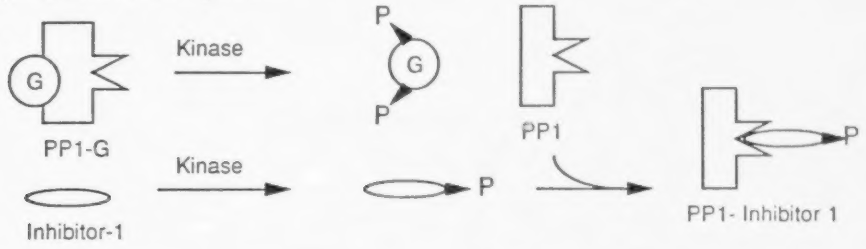

A.
Glycogen Chain
Glucose
Molecule
Kinase
Phosphatase
P
Glycogen Phosphorylase
OFF
Glycogen Phosphorylase
ON
B.
Kinase
Phosphatase
P
Glycogen Synthase
OFF
Glycogen Synthase
ON

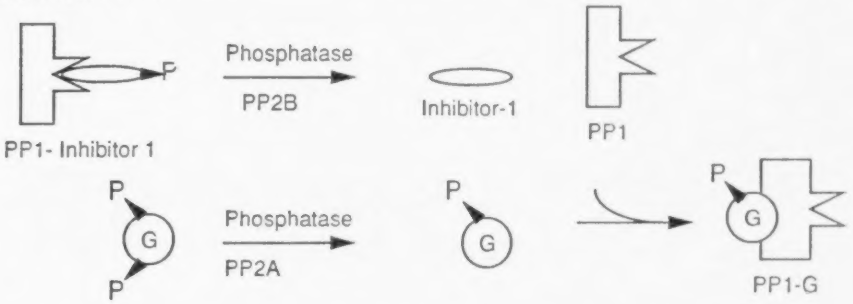
Figure 2.2. A simplified scheme illustrating the control of glycogen metabolism by reversible phosphorylation.
A) Phosphorylation activates the enzyme that cleaves glucose molecules from glycogen, (glycogen phosphorylase), resulting in the breakdown of glycogen. B) Phosphorylation deactivates the enzyme that synthesises glycogen from glucose. Protein kinases add phosphates and protein phosphatases cleave them off.


A
cAMP
Protein Kinases are Activated

1. Adrenaline (A), binds to the cell and stimulates an increase in the second messanger cyclic AMP. Cyclic AMP activates protein kinases. Protein kinases phosphorylate glycogen phosphorylase and glycogen synthase, see Figure 2.2.


G
PP1-G
Kinase
P
G
P
PP1
Kinase
Inhibitor-1
P
P
PP1- Inhibitor 1

2. Protein kinases "turn off" PP1 by phosphorylating the G-subunit and inhibitor-1. The phosphorylated G-subunit disassociates from PP1. Phosphorylated inhibitor-1 binds PP1 and deactivates PP1.


P
PP1- Inhibitor 1
Phosphatase
PP2B
Inhibitor-1
PP1
P
G
P
Phosphatase
PP2A
P
G
P
G
PP1-G

3. Protein phosphatases "turn on" PP1 by cleaving phosphates from inhibitor-1 and the G-subunit. PP1-G "turns off" energy production by dephosphorylating the glycogen enzymes, see Figure 2.2.

Figure 2.3 A simplified scheme depicting the activation of glycogen metabolism by adrenaline and deactivation by protein phosphatases.

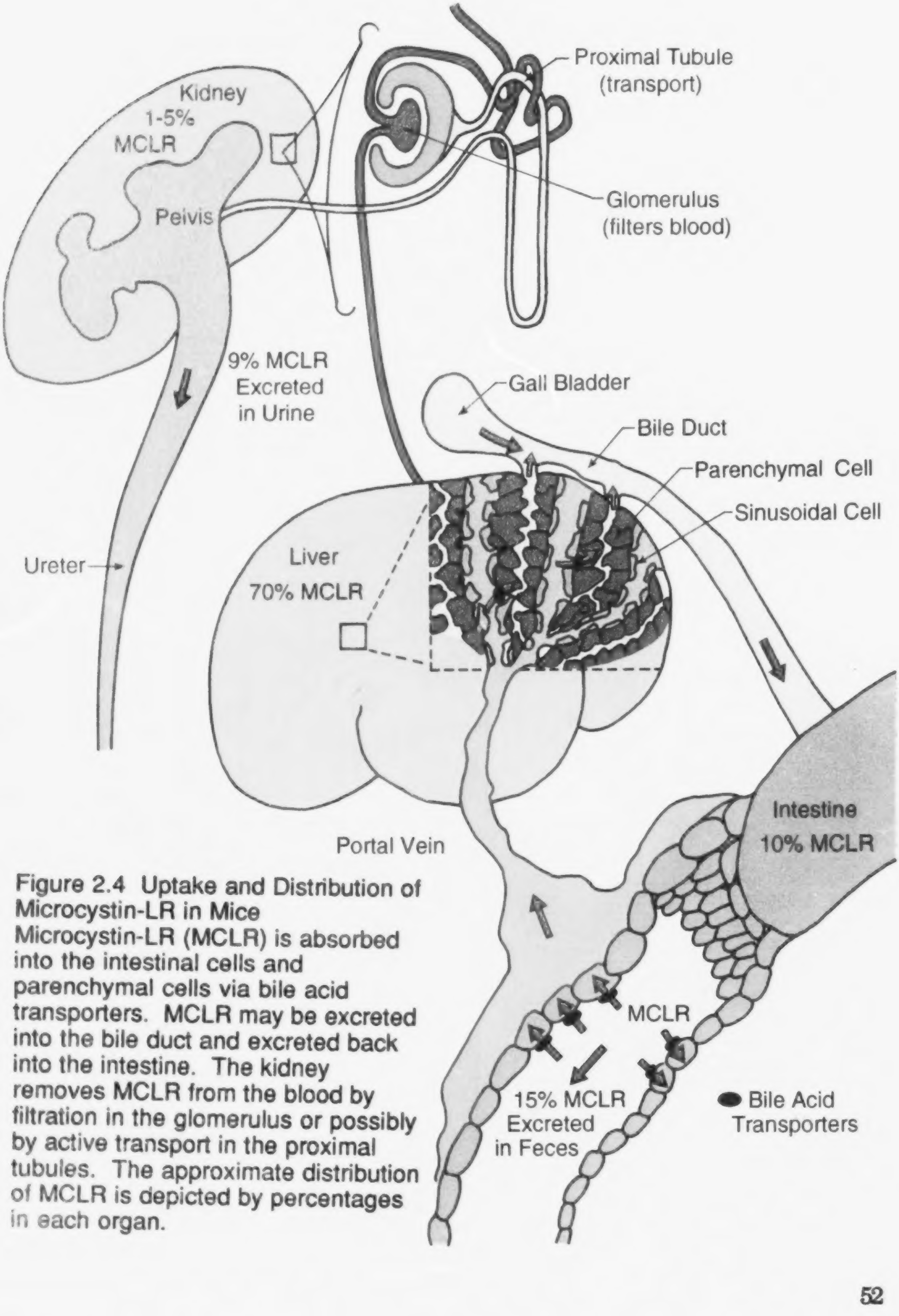

Proximal Tubule
(transport)
Kidney
1-5%
MCLR
Pelvis
Glomerulus
(filters blood)
9% MCLR
Excreted
in Urine
Gall Bladder
Bile Duct
Parenchymal Cell
Sinusoidal Cell
Ureter
Liver
70% MCLR
Intestine
10% MCLR
Portal Vein
MCLR
15% MCLR
Excreted
in Feces
● Bile Acid
Transporters

Figure 2.4 Uptake and Distribution of Microcystin-LR in Mice
Microcystin-LR (MCLR) is absorbed into the intestinal cells and parenchymal cells via bile acid transporters. MCLR may be excreted into the bile duct and excreted back into the intestine. The kidney removes MCLR from the blood by filtration in the glomerulus or possibly by active transport in the proximal tubules. The approximate distribution of MCLR is depicted by percentages in each organ.

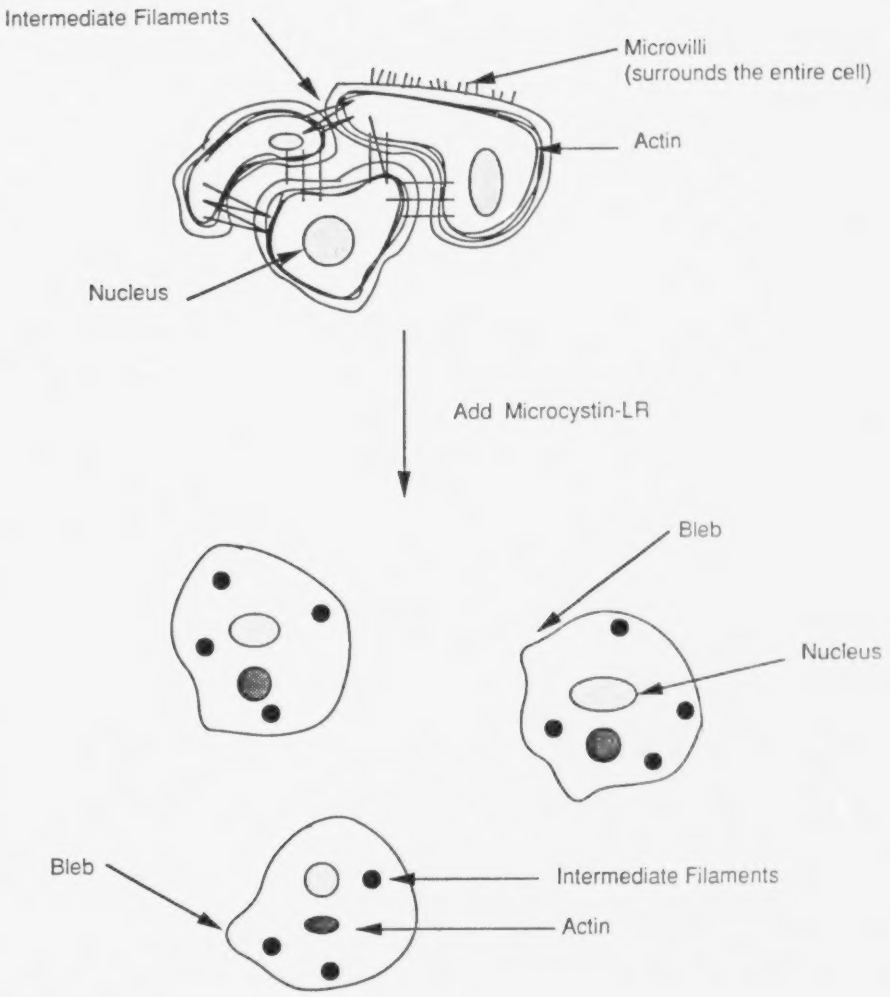

Intermediate Filaments
Microvilli
(surrounds the entire cell)
Actin
Nucleus
Add Microcystin-LR
Bleb
Nucleus
Bleb
Intermediate Filaments
Actin

Figure 2.5 Redistribution of the cell cytoskeleton in response to microcystin-LR. The actin filaments are located at the periphery of the cell and the intermediate filaments at places of cell to cell adhesion. Microvilli surround each cell. After absorption of microcystin-LR, the cells are observed to become round. The actin filaments are observed as a condensed ball-like structure in the center of the cell, and the intermediate filaments are observed as several clusters around the cell. Microvilli are observed to be lost from the cells, and blebs, or blisters appear on the surface of the cells. (Eriksson et al. 1989b, Falconer and Yeung 1992, Hooser et al. 1991a, Ohata et al. 1992, and Runnegar and Falconer et al. 1986).

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3.0 QUANTITATION OF THE MICROCYSTIN HEPATOTOXINS IN WATER AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS WITH THE PROTEIN PHOSPHATASE BIOASSAY

3.1 INTRODUCTION

The cyanobacterial microcystin toxins have been shown to be very potent hepatotoxins causing death to a variety of animals, reviewed (Carmichael 1992). The microcystins have also been shown to be potent tumor promoters (Nishiwaki-Matsushima et al. 1992, Falconer 1991). Toxic cyanobacteria (blue-green algae) blooms have been documented in drinking water sources in Finland, (Namikoshi et al. 1992), England and Europe (Lawton and Codd, 1991), China (Carmichael et al. 1988), Australia (Falconer et al. 1983c), and western Canada (Carmichael and Gorham 1981, Kenefick et al. 1992, Kotak et al. 1993). The ability to predict the toxicity of a bloom has not been established, as variations in toxicity change over a short period of time and spatially within the bloom itself (Carmichael and Gorham 1981). Toxicity assessments of cyanobacteria blooms by mouse bioassay indicate that about 50% of all cyanobacteria blooms are toxic (Carmichael and Gorham 1981, Kotak et al.1993, Lawton and Codd 1991, Repavich et al. 1990, Sivonen et al. 1990, Watanabe et al. 1991). Therefore toxicity analysis of bloom material is not indicative of microcystin concentrations that may be present in the drinking water.

The general structure of the microcystin toxins is cyclo(-D-Ala-L-X-D-*erythro-B*-methyl-Asp-L-Z-Adda-D-Glu-N-methyldehydro-Ala); where X and Z represent two variable L amino acids, (leucine, arginine, tyrosine, alanine, or methionine); and Adda (2S,3S,8S,9S,)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid is the unique C_{20} amino acid so far found only in these cyanobacterial peptide toxins (Botes et al 1985). Microcystin-LR is perhaps the best known algal toxin (Figure 3.1), where the X is leucine and the Z is arginine, but there are a great number of other variations (Carmichael 1992).

Several incidents of human illness have been attributed to the presence of the algal toxins in the drinking water in Australia, Africa, and the United States (Tisdale 1930, Zilberg 1966, Bourke et al. 1983, Falconer et al. 1983a). Water treatment studies conducted at the laboratory and pilot plant scale have concluded that activated carbon and ozone are capable of removing microcystins from drinking water below the detection limit of the mouse bioassay and High Performance Liquid Chromatography with ultraviolet light detection (HPLC/UV) (Wheeler et al. 1942, Hoffman 1976, Himberg et al. 1989, Falconer et al. 1983b and 1989, and Keijola et al. 1988). Conventional water treatment practices (coagulation/sedimentation, filtration, and chlorination), have been found to be ineffective at removing the toxins (Wheeler et al.1942, Hoffman 1976, and Himberg et al.1989). Because of the lack of a sensitive analytical technique, the water treatment studies have been conducted at concentrations higher than would likely be encountered at treatment facilities. Studies in our laboratory have demonstrated the presence of a residual concentration of microcystin-LR after both GAC and PAC processes in full-scale treatment plants (section 4).

Microcystin-LR has been shown to be a potent inhibitor of serine/threonine protein phosphatases (PP1 and PP2A) and this probably underlies it's toxicity to animals (Eriksson et al. 1990, Honkanen et al. 1990, MacKintosh et al. 1990). Microcystin-LR inhibited either PP1 or PP2A at concentrations of ~0.1 nM when assays were performed at phosphatase concentrations of 0.2 mUnits/mL (MacKintosh et al. 1990). The IC_{50} values for inhibition of PP1 and PP2A by microcystin-LR were found to depend on the concentration of phosphatase in the assay (MacKintosh et al. 1990). This paper presents an application of the protein phosphatase (PP) bioassay (Holmes 1991) for quantification of microcystin toxins in drinking water at environmental concentrations. Standard curves for microcystin-LR at various concentrations of PP1 were prepared. The dependence of microcystin-LR inhibition of PP1 activity in the assay upon the concentration of PP1 used in the assay was evaluated. The PP bioassay was used to quantify microcystin in raw water and finished drinking water at concentrations as low as 0.1 µg/L microcystin-LR (section 4).

3.2 METHOD AND MATERIALS

Enzymes and reagents were prepared as described in Holmes (1991). PP1 was purified from rabbit skeletal muscle, and stored in a 60% (v/v) glycerol solution at - 4 °C. The protein phosphatase bioassay was performed as described in Holmes (1991). 10 µL protein phosphatase, PP1 (1 mU/mL) and a 10 µL standard of varying concentration of microcystin-LR in TRIS HCl buffer (50 mM, pH 7.0) were incubated at 30 °C for 10 minutes. A 10 µL sample of ^{32}P-radiolabelled phosphorylase a (3.5 mg/mL in TRIS HCl pH 7.0) was added to start the reaction and the mixture incubated at 30 °C for 10 min. The reaction was stopped by adding 200 µL of 20% (w/v) TCA and the samples placed in ice for two min. The samples were centrifuged for 2 min and 200 μL of the supernatant was added to 1 mL of scintillation fluid (ACS). The cleaved ^{32}P-radiolabelled phosphate was counted with a Pharmacia 1209 Rackbeta Liquid Scintillation Counter and IBM Proprinter.

The ^{32}P-phosphorylase a was prepared by incubating phosphorylase b (25 mg/L), phosphorylase kinase (Sigma, 0.2 mg/mL), magnesium acetate (2.8 mM), EDTA (0.25 mM), calcium chloride (0.125 mM), sodium glycerophosphate (100 mM), TRIS HCl 125 mM, pH 8.6), ^{32}P -ATP (125 μCi) and ATP (1 mM) at 30 °C for one hour. The reaction was stopped by adding ice cold 70% saturated ammonium sulphate (pH 8.0), and the mixture placed on ice for 15 min. The reaction mixture was then centrifuged at 27000 g for 10 min and the supernatant discarded. The pellet was resuspended in a minimum volume of TRIS HCl (50 mM, pH 7.0), (EDTA 1 mM), 0.1% (v/v) 2-mercaptoethanol, sodium fluoride (25 mM) and dialysed against the same buffer to remove ^{32}P-ATP and ^{32}P-inorganic phosphate. The prepared phosphorylase a was stored as a crystalline suspension and stored at 4 °C.

Standard microcystin-LR was obtained from Calbiochem (San Diego, California). The purity of the standard was assessed by HPLC, with a Vydac C-18 analytical column, 10 mM ammonium acetate/acetonitrile mobile phase. The concentration of the standard was assessed by amino acid analysis. A stock solution of 5 pg/μL standard microcystin -LR was prepared. Standard solutions 4 pg/μL, 3 pg/μL and 2 pg/μL were prepared with fresh TRIS HCl buffer (50 mM, pH 7.0) buffer for each assay. TRIS HCl buffer consisted of; 50 mM TRIS HCl pH 7.0, 0.1 mM EDTA, 1 mg/mL BSA, 0.2% V/V 2- mercaptoethanol, and 1.5 mM caffeine.

3.3 CALCULATION OF MICROCYSTIN-LR CONCENTRATION

The inhibition of PP1 and PP2A by microcystin-LR has been shown to have a sigmoidal response curve when plotting % activity of PP1 vs log microcystin-LR concentration (Eriksson et al.1990, Honkanen et al.1990, and MacKintosh et al.1990). The curve is linear in the 50% PP1 activity range and therefore microcystin-LR sample concentrations can be quantified in this region of PP1 activity (Figure 3.2). Standard solutions of microcystin-LR were used to calculate the equation of the linear portion of the PP1 activity curve. Water samples were diluted or concentrated such that they inhibit PP1 activity by approximately 50%.

The PP1 activity of all samples, microcystin-LR standard and water sample were calculated by the equation;

$$\%\ \text{Activity} = 100 - \frac{(\text{control - blank}) - (\text{sample - blank})}{(\text{control - blank})} \times 100\%$$

where

control counts is the maximum PP1 Activity;

control = maximum activity of the PP1 enzyme, 100% activity

10 μL TRIS HCL buffer

10 μL phosphatase (1 U/mL) PP1

10 μL phosphorylase a

blank or background counts;

blank = free ^{32}P - phosphate in the phosphorylase a solution

20 μL TRIS HCL buffer
0 μL phosphatase (1 U/mL) PP1
10 μL phosphorylase a

microcystin-LR standard counts;

10 μL of standard microcystin-LR in TRIS HCL buffer
10 μL phosphatase (1 U/mL) PP1
10 μL phosphorylase a

water sample counts;

10 μL = 1-3 μL sample + 7-9 μL TRIS HCL buffer
10 μL phosphatase (1 U/ml) PP1
10 μL phosphorylase a

The amount of inhibition of PP1 and PP2A by a standard concentration of microcystin-LR depends on the concentration of phosphatase in the assay (MacKintosh et al. 1990). The amount of enzyme in the assay can be related to the amount of ^{32}P released from phosphorylase a during the assay relative to the total amount of ^{32}P present. The % release is calculated by the equation,

$$\% \text{ release} = \frac{(\text{control counts - blank counts})}{(\text{total counts - blank counts})} \cdot 1.15 \cdot 100$$

where

Total counts, the concentration of ^{32}P in the substrate;
total = ^{32}P-phosphorylase a and free ^{32}P-phosphate
10 µL substrate + 200 µL scint. fluid

and 1.15 is a dilution factor;

$$= \frac{\text{volume of reaction contents counted}}{\text{total reaction contents in assay.}}$$

The change in PP1 activity with % release was evaluated by plotting % activity vs % release at 2, 3, and 4 µg/L microcystin-LR.

3.4 RESULTS AND DISCUSSION

The HPLC profile of the microcystin-LR standard was observed as a single peak. The amino acid analysis confirmed the concentration of the standard to be 2 µg/µL. The PP1 isolated from rabbit skeletal muscle was potently inhibited by microcystin-LR standard in the assay (Figure 3.2) in a manner previously described (Eriksson et al. 1990, MacKintosh et al. 1990, and Honkanen et al. 1990).

As the concentration of enzyme increased in the assay, the amount of inhibition observed with a standard concentration of microcystin-LR decreased. Standard curves at various concentrations of enzyme (% release) are illustrated in Figure 3.3. For example, a change from 45 to 75 % PP1 activity was observed with increases from 11 to 27 % release of ^{32}P from labelled phosphorylase a. The change in inhibition was observed at 2, 3 and 4 pg/µL microcystin-LR. The relationship between PP1 activity and % release was evaluated by linear regression at 2, 3, and 4 pg/µL microcystin-

LR in the assay (Figure 3.4). The slope at each concentration was approximately the same for all three concentrations, ~1.5 (% PP1 activity)/(% release). Fresh solutions of PP1 enzyme and ^{32}P-phosphorylase a are prepared for each assay and therefore % release values were observed to vary from one assay to another. To account for the variation in PP1 activity, a standard microcystin-LR curve was established in each assay.

For the evaluation of water samples, the sample was diluted or concentrated so that the activity of PP1 was within the bounds of the microcystin-LR standard curve. The water samples required no preparation before analysis and a collected sample volume of 5 mL was adequate for quantitation. Reproducible results were obtained with both raw and treated samples suggesting that natural organic matter present in the samples did not interfere with the assay (Tables 3.1 to 3.5)

A large number of microcystin analogues have been observed in a bloom (Namikoshi et al. 1992). The PP assay does not distinguish between microcystin analogues, and measures the PP1 inhibition of the water as whole. The inhibition of PP1 by microcystin-LR, microcystin-RR, and nodularin differs for each toxin (Eriksson et al.1990). The effect of varying concentrations of microcystin analogues has not been evaluated. Therefore the quantity of toxin in the water is reported in units of microcystin-LR equivalents per volume. Because the toxicity of the microcystins is probably through inhibition of protein phosphatases (Eriksson et al.1990), the measured toxicity of the water with the PP assay is representative of the toxicity to biological systems.

With the PP assay, the concentration of microcystin toxins can be evaluated at water concentrations relevant to environmental conditions. Mycrocystin-LR was quantified in drinking water at 0.1 µg/L (section 4). The assay represents a means of identifying the presence of microcystin toxin in drinking water before concentrations increase to hazardous levels. With careful monitoring the health risk associated with drinking water containing microcystins can be minimized and the likelihood of a serious exposure incident reduced.

Table 3.1 Camrose Raw and Treated Total Microcystin Concentrations.

Water Sample	Total Microcystin Average	 Standard Deviation
Raw	(μg/L microcystin-LR equivalents)	
26-Aug	0.15	0.04
27-Aug	0.87	0.27
31-Aug	0.86	0.08
2-Sep	0.67	0.16
4-Sep	0.12	0.01
8-Sep	0.62	0.08
10-Sep	0.73	0.10
14-Sep	0.35	0.07
16-Sep	0.29	0.03
18-Sep	0.26	0.07
22-Sep	0.27	0.03
24-Sep	0.15	0.03
28-Sep	0.17	0.03
30-Sep	0.22	0.07
2-Oct	0.15	0.02
Treated		
26-Aug	0.11	0.03
27-Aug	0.09	0.03
31-Aug	0.13	0.05
2-Sep	0.10	0.04
4-Sep	0.14	0.03
8-Sep	0.18	0.03
10-Sep	0.17	0.01
14-Sep	0.15	0.04
16-Sep	0.14	0.05
18-Sep	0.14	0.03
22-Sep	0.18	0.08
24-Sep	0.10	0.04
28-Sep	0.14	0.05
30-Sep	0.15	0.03
2-Oct	0.14	0.03

Table 3.2 Ferintosh Treatment Data, August 25/92

Sample	Time	Average	Standard Deviation
		(µg/L microcystin-LR)	
Tap-3	12:00	0.54	0.22
T-4	13:00	0.50	0.22
T-5	14:00	0.55	0.22
T-6	15:00	0.64	0.20
T-7	16:00	0.63	0.19
T-8	17:00	0.31	0.10
Carbon-1	10:00	0.42	0.07
C-2	11:00	0.39	0.10
C-3	12:00	0.46	0.12
C-4	13:00	0.48	0.13
C-5	14:00	0.48	0.02
C-6	15:00	0.67	0.07
C-7	16:00	0.53	0.06
C-8	17:00	0.52	0.14
Filter-1	10:00	0.61	0.20
F-2	11:00	0.86	0.23
F-3	12:00	0.68	0.04
F-4	13:00	0.86	0.48
F-5	14:00	0.63	0.23
F-6	15:00	1.85	0.17
F-7	16:00	2.01	0.30
F-8	17:00	2.32	0.21
Coag-1	10:00	2.44	0.75
Co-2	11:00	1.37	0.46
Co-3	12:00	1.78	0.54
Co-4	13:00	1.79	0.59
Co-5	14:00	2.67	0.99
Co-6	15:00	3.92	1.62
Co-7	16:00	5.38	1.93
Co-8	17:00	5.31	2.20
Raw-1	10:00	2.79	0.64
R-2	11:00	2.84	1.34
R-3	12:00	4.26	1.31
R-4	13:00	2.77	0.71
R-5	14:00	3.07	0.65
R-6	15:00	2.44	0.61
R-7	16:00	2.89	0.25
R-8	17:00	2.28	0.89

Table 3.3 Ferintosh Treatment Data, October 1/92

	TIME	Average (µg/L microcystin-LR)	Standard Deviation
Tap-1	12:00	0.38	0.05
T-2	13:00	0.28	0.07
T-3	14:20	0.37	0.04
T-4	15:00	0.32	0.09
T-5	16:00	0.35	0.02
T-6	17:30	0.31	0.08
T-7	18:30	0.31	0.09
T-8	19:30	0.36	0.01
T-9	20:30	0.37	0.06
Carbon-1	12:00	0.24	0.03
C-2	13:00	0.32	0.18
C-3	14:20	0.31	0.04
C-4	15:00	0.32	0.07
C-5	16:00	0.27	0.03
C-6	17:30	0.42	0.05
C-7	18:30	0.50	0.02
C-8	19:30	0.34	0.05
C-9	20:30	0.34	0.02
Filter-1	12:00	0.39	0.01
F-2	13:00	0.76	0.04
F-3	14:20	0.62	0.08
F-4	15:00	0.81	0.02
F-5	16:00	0.41	0.12
F-6	17:30	0.42	0.11
F-7	18:30	0.67	0.19
F-8	19:30	0.77	0.07
F-9	20:30	0.58	0.03
Coag-1	12:00	1.27	0.15
Co-2	13:00	0.45	0.02
Co-3	14:20	0.87	0.03
Co-4	15:00	0.78	0.04
Co-5	16:00	0.38	0.04
Co-6	17:30	0.56	0.02
Co-7	18:30	0.59	0.03
Co-8	19:30	0.67	0.03
Co-9	20:30	0.76	0.09
Raw-1	9:00	1.89	0.23
R-2	10:00	0.54	0.03
R-3	11:00	0.83	0.06
R-4	12:00	0.52	0.11
R-5	13:00	0.57	0.01
R-6	14:20	0.97	0.01
R-7	15:00	1.00	0.02
R-8	16:00		
R-9	17:30	0.45	0.03
R-10	18:30	2.41	0.30
R-11	19:30	1.83	0.22
R-12	20:30	1.69	0.21

Table 3.4 Little Beaver Lake and Tap Water Samples

	Lake Water	Tap Water
	(µg/L Microcystin-LR)	
27-May	0.12	0.18
2-Jun	0.05	0.13
9-Jun	1.35	0.11
15-Jun	2.28	0.1
24-Jun	0.98	0.11
30-Jun	1.43	0.05
7-Jul	1.31	0.12
14-Jul	0.27	0.11
21-Jul		0.09

Table 3.5 Driedmeat Lake Water Samples.

	Lake Water (µg/L Microcystin-LR)
27-May	0.05
2-Jun	0.06
9-Jun	0.07
15-Jun	0.06
24-Jun	0.19
7-Jul	0.07
14-Jul	0.01

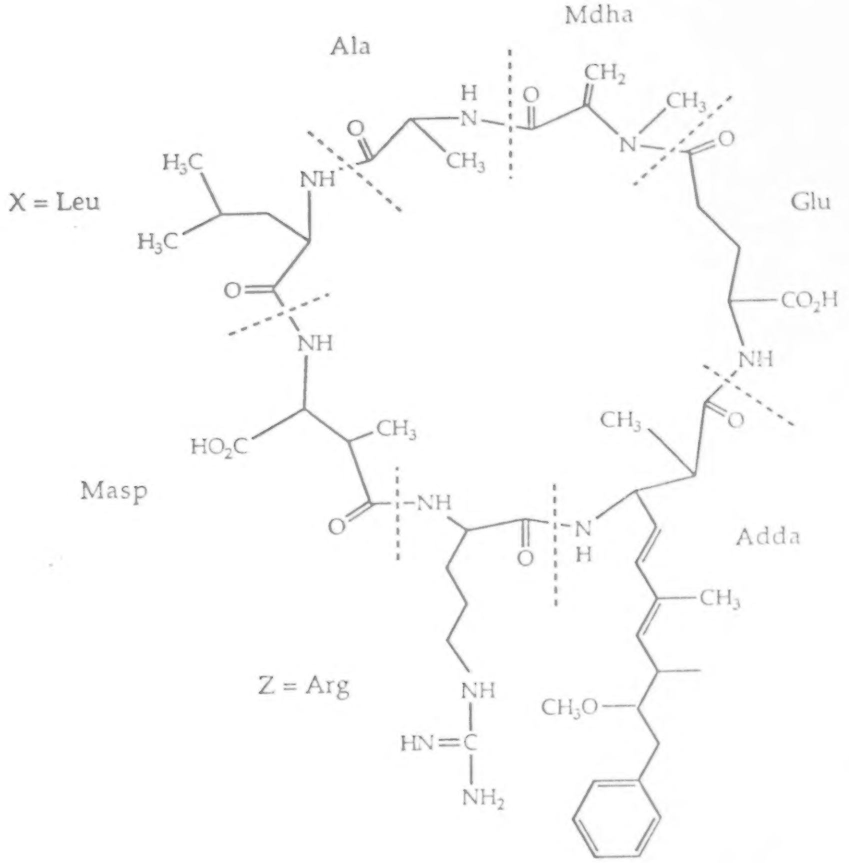
Mdha
Ala
X = Leu
Glu
Masp
Adda
Z = Arg
H
N
O
CH2
CH3
NH
H3C
CO2H
HO2C
CH3O
HN=C
NH2

Figure 3.1 Chemical Structure of Microcystin-LR.

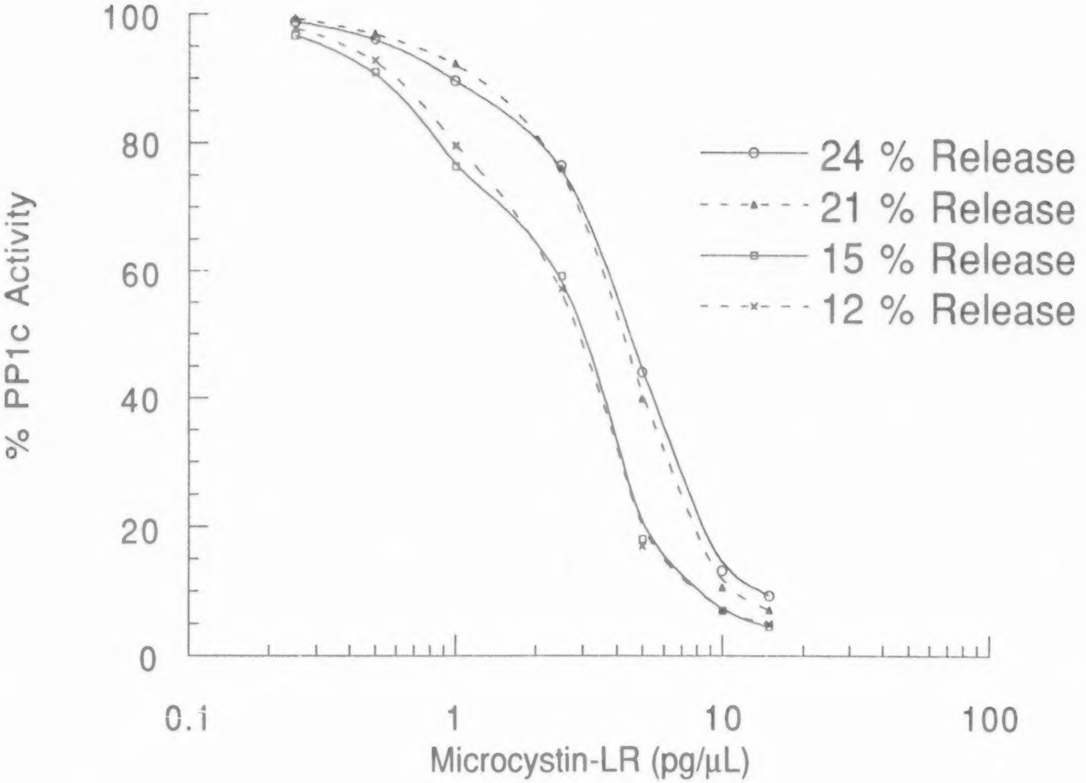

Figure 3.2 PP1c Activity Profile In The Presence of Microcystin-LR.

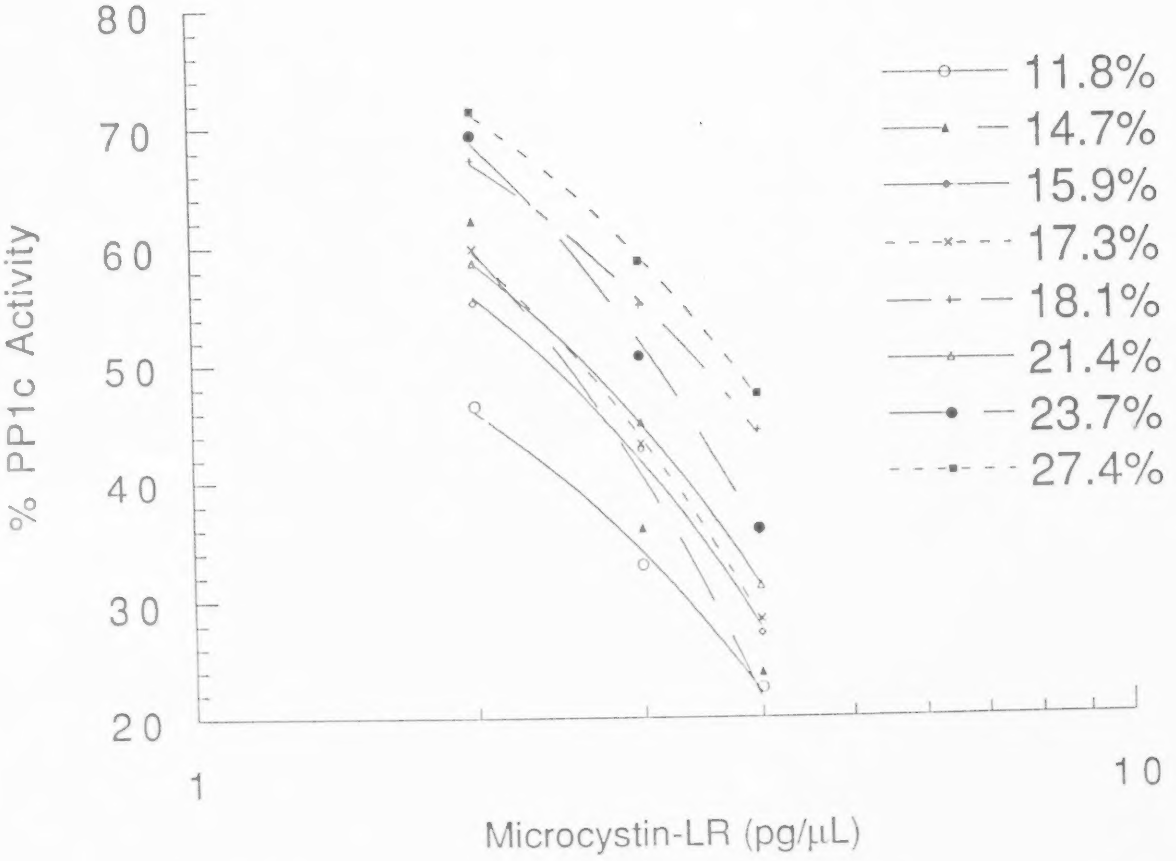

Figure 3.3 Microcystin-LR Standard Curves for the PP Bioassay.

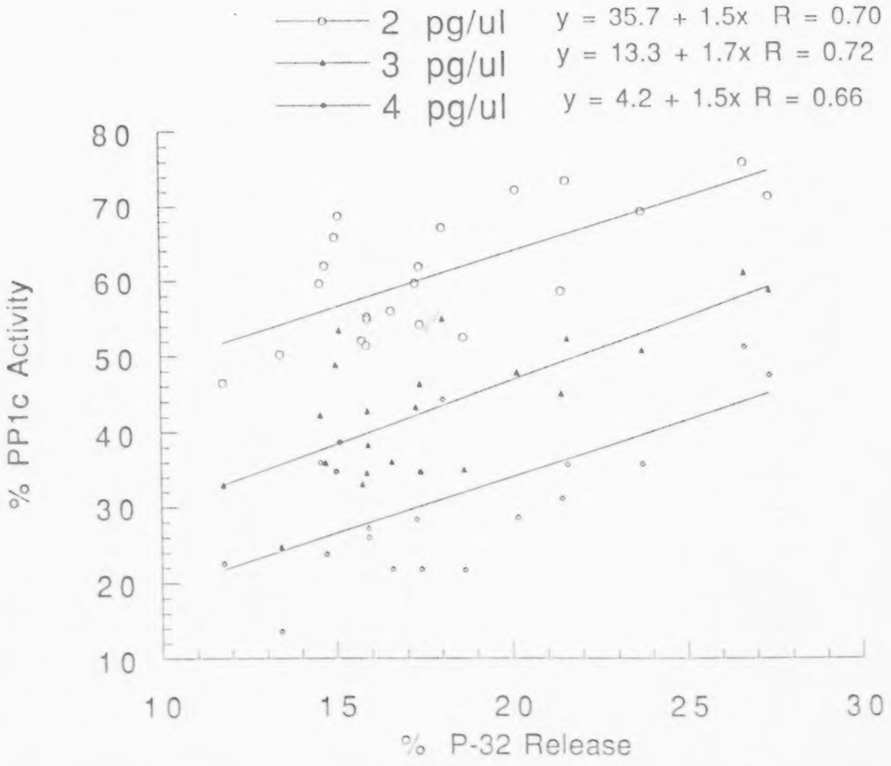

Figure 3.4 Change in PP1c Activity with ^{32}P % Release at Various Concentrations of Microcystin-LR.

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4.0 OCCURRENCE AND REMOVAL OF MICROCYSTIN-LR IN DRINKING WATER TREATMENT

4.1 INTRODUCTION

The microcystin algal toxins produced from cyanobacteria are becoming a greater concern around the world as the occurrence of toxic cyanobacteria blooms becomes recognized and eutrophication of drinking water sources continues. The microcystins have been found to be extremely potent hepatotoxins and are lethal at high concentrations to many different animals (Carmichael 1992). The microcystins have also been shown to be very powerful tumor promoters (Nishiwaki-Matsushima et al. 1992, Falconer 1991). Several incidents of human illness have been attributed to the presence of cyanobacteria in drinking water although there has been limited documentation obtained in these incidents (Tisdale 1930, Zilberg 1966, Bourke et al. 1983, Falconer et al. 1983a). Despite substantial documentation that microcystin-LR can be found within algal bloom cell biomass collected from drinking water supplies (Kenefick et al. 1992, Kotak et al. 1993), there has been doubt expressed by regulatory authorities about the occurence and levels of microcystins in raw and treated drinking waters.

Conventional water treatment practices (coagulation/sedimentation, filtration, and chlorination), have been found to be ineffective at removing the toxins (Wheeler et al. 1942, Hoffman 1976, and Himberg et al. 1989). Water treatment studies conducted at the laboratory and pilot plant scale have concluded that granular activated carbon (GAC) filtration is effective in removing the algal toxins from drinking water (Hoffman 1976, Falconer et al. 1983b and 1989, Himberg et al. 1989, Keijola et al. 1988). Powdered activated carbon (PAC) was shown to remove the toxins but at doses higher than are generally used at water treatment facilities (Wheeler et al. 1942, Hoffman 1976, Falconer et al. 1983b, Himberg et al. 1989 and Keijola et al. 1988). Ozone has also been shown to be effective at removing the toxicity in laboratory and pilot plant studies (Himberg et al. 1989, Keijola et al. 1988). The water treatment studies and the health effects associated with microcystin-LR has been reviewed in section 2.

The water treatment studies conducted thus far have been at concentrations much higher than would likely be encountered in raw water supplies. The use of high toxin concentrations may overestimate the removability of toxin by activated carbon especially in the presence of background organic matter (Najm et al. 1991, Qi et al. 1992). High concentrations, have been used in the experiments because there has been no analytical technique available to measure microcystin at realistic raw water concentrations. The published studies have employed the mouse bioassay or High Performance Liquid Chromatography with Ultraviolet Light Detection (HPLC/UV) for toxin determination. Likewise, the identity of the algal toxins used in the experiments was not determined but they generally were confirmed to be hepatotoxic by dosing of experimental animals.

The present work focuses on the fate and identification of the microcystin toxins in water treatment processes at environmental concentrations. There is a large family of microcystin analogues produced by cyanobacteria, reviewed (Carmichael 1992). The general structure of the microcystin toxins is cyclo(-D-Ala-L-X-D-*erythro-B*-methyl-Asp-L-Z-Adda-D-Glu-N-methyldehydro-Ala); where X and Z represent two variable L amino acids, (leucine, arginine, tyrosine, alanine, or methionine); and Adda (2S,3S,8S,9S,)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid is the unique C_{20} amino acid so far found only in these cyanobacterial peptide toxins (Botes et al 1985). The structure of Microcystin-LR is illustrated (Figure 4.1) where the X is leucine and the Z is arginine. The protein phosphatase (PP) bioassay (Holmes 1991) was applied to identify and quantify the concentration of microcystin in water at environmental concentrations. The PP bioassay does not distinguish between microcystin analoques but measures the total quantity of microcystins in the water reported as microcystin-LR equivalents. Therefore the major microcystin analogue in the water causing phosphatase inhibition in the PP bioassay was purified and identified.

The removals of microcystin by conventional processes and GAC filtration at the Ferintosh water treatment facility were evaluated (Figure 4.2). Raw and treated concentrations of total microcystins at the Camrose treatment facility utilizing conventional processes with PAC were determined over the month of September (Figure 4.3). Sorption isotherms were determined for pure microcystin-LR using virgin and pre-loaded carbon in buffered lab grade water. Pre-loaded activated carbon was removed from the Ferintosh treatment facility GAC bed where it has been for at least 5 months. Competitive sorption isotherms were determined by using water removed immediately before entering the GAC filter at the same facility rather than with buffered lab grade water.

4.2 METHODS AND MATERIALS

4.2.1 Protein Phosphatase Bioassay

The PP bioassay was conducted as described in Holmes (1991) with a few variations. A standard curve with pure microcystin-LR purchased from Calbiochem (San Diego, California) was used for quantitation of samples. The expected concentration of the standard was evaluated by amino acid analysis. The purity of the standard was assessed by the absence or presence of other HPLC peaks. Standard samples of microcystin-LR were included in every PP bioassay to account for variations in the activity of protein phosphatase. A detailed description of the PP bioassay is described in section 3.

4.2.2 Microcystin-LR Purification and Identification

A Dionex gradient mixer HPLC pump equipped with a Waters 484 Tunable Absorbance detector, and a Dionex 4400 integrator were used for the purification and identification of microcystin-LR in the drinking water.

4.2.2.1 Purification from Drinking Water

A 1 L sample of the water collected from the Ferintosh treatment train (just before entering the carbon bed) was evenly split into four 250 mL round bottom flasks and placed on a Labconco freeze drier model 4.5. The dried residues were then extracted 5 times with methanol. The methanol fractions were pooled and dried down under a stream of nitrogen. The sample was reconstituted in 1 mL of 10 mM ammonium acetate/acetonitrile (85:15), pH 6.5. The sample was loaded onto a Vydac C-18 analytical column. The mobile phase started at 85:15 and increased in polarity to 90:10 10 mM ammonium acetate/acetonitrile over 45 min, at a flow rate of 1 mL/min to separate the microcystin-LR from background organic matter. The microcystin-LR sample was then eluted by starting with 10 mM ammonium acetate /acetonitrile (85:15) for 10 min, followed by a gradient, 85:15 to 70:30 over 30 min, with a flow rate of 1 mL/min. One mL fractions were collected and assayed with the PP bioassay. The peak fractions corresponding to microcystin-LR were pooled and dried down under a stream of nitrogen. The approximate retention time was determined with a standard sample of microcystin-LR. A blank gradient was run and analyzed by the PP bioassay before sample application to ensure that there was no residual microcystin-LR remaining on the column.

4.2.2.2 Identification of Microcystin-LR

The pool of microcystin-LR sample was reconstituted in 1 mL of 0.1% trifluoroacetic (TFA) acid in water:acetonitrile (80:20), pH 2.0. The fraction was loaded on the Vydac C-18 analytical column, and eluted by starting with 0.1% TFA/acetonitrile (80:20) for 15 min, followed by a gradient from 80:20 to 60:40 over 60 min, with a flow rate of 1 mL/min. One mL fractions were collected and assayed with the PP bioassay. The peak fractions corresponding to microcystin-LR were pooled and dried down under a stream of nitrogen. The pooled fractions were reconstituted in 1 mL of 10 mM ammonium acetate:acetonitrile (85:15), pH 6.5. The microcystin-LR sample was loaded onto a Vydac C-18 analytical column, and eluted by starting with 10 mM ammonium acetate/acetonitrile (85:15) for 10 min, followed by a gradient from 85:15 to 70:30 over 30 min, with a flow rate of 1 mL/min. One mL fractions were collected and assayed with the PP bioassay to determine the peak fractions.

The peak fractions were pooled, dried down under nitrogen and reconstituted in 80 µL of 10 mM ammonium acetate/acetonitrile (76:24), pH 6.5. Half of the sample was run isocratically, 10 mM ammonium acetate/acetonitrile (76:24). A 50:50 mixture (wt/wt) of standard microcystin-LR and the Ferintosh sample was run under the same conditions. A standard microcystin-LR sample of approximately the same concentration was also run isocratically.

Before each HPLC run a standard sample of microcystin-LR was run on the same gradient to determine the elution time. A blank gradient was then run after each standard to insure that no residual microcystin-LR was left on the column. The standard and blank fractions were collected and assayed with the PP bioassay before the sample was injected onto the column.

4.2.3 Fate of Microcystin-LR in Water Treatment Processes

Samples were withdrawn every hour from each stage of the Ferintosh treatment train (Figure 4.2) on August 25 and October 1 1992. Four 1 mL samples were collected in eppendorf tubes and a 500 mL sample for total organic carbon (TOC) analysis. The TOC samples were acidified immediately with o-orthophosphoric acid to a pH less than 2. All of the samples were placed in a cooler for storage until reaching the lab. The TOC samples were analyzed with a Dohrman Carbon Analyzer (model 4) in triplicate as per standard methods TOC analysis with one exception. A small amount of precipitation occurred after acidifying samples with o-orthophosphoric acid and therefore samples were homogenized with a

Brinkman Polytron before analysis. Each sample was purged with helium to remove inorganic carbon before analysis. The eppendorf samples were stored at - 4 °C until analysis. Each sample was analyzed in triplicate with the PP bioassay.

Raw and treated water samples were collected from the Camrose treatment train (Figure 4.3) and analyzed by the PP bioassay. Raw water samples were collected after the microscreen at the raw water intake, and treated water was collected from a drinking water tap. The samples were collected between 11 AM and 12 noon. The samples were stored at - 4 °C until they were analyzed.

4.2.4 Sorption Isotherms

4.2.4.1 Activated Carbon

Three commercial types of activated carbon were evaluated with sorption isotherms. Haycarb (pre-loaded) carbon samples were removed from the top of the GAC bed at the Ferintosh treatment facility. Haycarb virgin carbon was obtained for comparison in competitive and virgin isotherms. Scanning electron microscope photographs of the pre-loaded and virgin carbon were taken to confirm the presence of a biofilm in the former. Norit 0.8 supra and Filtrasorb F-100 were also evaluated in virgin and competitive isotherms because they were found to be the most effective by Falconer et al. (1983b and 1989).

The carbon was crushed using a SPEX 2000 Mixer/Mill, based on the typical carbon crushing standards (Andrews 1990). The mixer/mill crushing cylinder was filled approximately half-full with carbon and sixty-four 6 mm chrome-plated balls. The carbon was crushed for 2 min. The carbon was then washed through a #200 (75.0 μm) sieve and retained on a #400 (39 μm) sieve. The carbon was meticulously washed with Milli-Q water through the sieves to remove carbon fines. The carbon was then dried at 103 °C for approximately 24 h, or until there was no apparent change in weight. The carbon was stored in glass jars, and equilibrated to room temperature in a desiccator. For pre-loaded isotherms, both crushed and uncrushed carbon was evaluated. The crushed carbon was prepared as discussed. The uncrushed carbon was dried at 103 °C for one day, and allowed to cool before use in the isotherms.

4.2.4.2 Water Matrices.

The virgin and pre-loaded isotherm water matrix was prepared by spiking Milli-Q water (buffered with 10 mM K_2HPO_4, adjusted to pH 7.8 with HCl) with standard microcystin-LR. The water matrix for the competitve isotherms was obtained just prior to entering the GAC bed at the Ferintosh treatment facility on October, at 16:00 h. The water was filtered through a Whatman # 2 filter to remove debris. The filtered water was spiked with standard microcystin-LR. The pH and Total Organic Carbon (TOC) were determined for the filtered water. The water obtained from Ferintosh was stored at 4 °C after collection.

Initial concentrations of ~1 μg/L to ~10 μg/L microcystin-LR were used in the isotherms. All solutions were mixed for 24 h before use in the isotherms.

4.2.4.3 Equilibrium Time

The equilibrium time for microcystin-LR adsorption was assessed by observing the change in microcystin-LR concentration in solution with time after contact with carbon. The time at which the concentration of microcystin-LR did not change with time was considered the equilibrium time. Seven samples of carbon, 0.5 g, virgin and pre-loaded (uncrushed) were placed in 160 mL bottles. Blank bottles and sample bottles were filled head space full with a 1.0 μg/L microcystin-LR solution prepared as discussed. A blank and sample bottle was removed each day and the concentration of microcystin-LR determined with the PP bioassay.

4.2.4.4 Isotherm Procedure

Sample of prepared activated carbon was accurately weighed in varying quantities into 160 mL glass bottles that had been acid washed and autoclaved. Each carbon weight was added in triplicate. The bottles were then filled head space full with a microcystin-LR solution and capped with teflon septum caps. The bottles were placed in a rotary tumbler, in a temperature controlled room, at 20 °C and rotated at 25 rpm for 24 h. The samples were then filtered through Whatman GF/F (non-binding) glass fibre filters to remove the carbon from solution. Four 1 mL aliquots were withdrawn from each filtrate and transferred to a 1 mL eppendorf tube and stored at -4 °C. A 1 mL aliquot of each sample was dried down in a Savant Speed Vac SC 100 (approximately 4 h) and resuspended in distilled water. The samples were then analyzed by the PP bioassay.

Blanks, bottles containing no carbon, were used to determine the initial concentration of the microcystin-LR solution. Blank bottles were filled every fourth sample and treated identically to the carbon samples at each step of the procedure.

The virgin carbon isotherm was prepared with 39 sample points, the pre-loaded carbon isotherm with 33 sample points and the competitive isotherm with 32 sample points. The isotherm data was analyzed by the Freundlich equation,

$$q = K_f \cdot C_f^{(1/n)},$$

where: $q = (C_i - C_f) \cdot$ Volume/mass carbon, giving the loading of the substance on the carbon in μg substance per g of carbon.

Cf is the equilibrium concentration of the substance in μg substance per L of solution.

Ci is the initial concentration of the substance in μg substance per L of solution.

K_f is a constant providing a rough approximation of the adsorptive capacity of the carbon and 1/n is a constant providing a rough approximation of the adsorption intensity.

Regression analysis was applied to untransformed data to determine the linear isotherm, and to calculate the 95% confidence limits for the estimated mean Y value for a given X_i, $\widehat{Y_i}$ value:

$$Y = \sqrt{S^2_{Y\cdot X}\left[\frac{1}{n}+\frac{(X_i-\overline{X})^2}{\Sigma x^2}\right]} \qquad \text{where } \eta = n - 2$$

and for the predicted Y_i for a given X_i value:

$$Y_i = \sqrt{S^2_{Y\cdot X}\left[1 + \frac{1}{n} + \frac{(X_i - \overline{X})^2}{\Sigma x^2}\right]} \qquad \text{where } \eta = n - 2.$$

The software packages Microsoft Excel, and Kaleidograph were used for data analysis and graphics.

4.2.5 Biodegradation Analysis

To determine if biodegradation contributes to microcystin-LR removal in the GAC bed, water samples from Ferintosh were equilibrated with 0.5 g of carbon from the Ferintosh facility GAC bed. One mL samples were withdrawn every few days and analyzed with the PP bioassay for 1 month. A control sample of autoclaved GAC, with 0.05% sodium azide added to the water to prevent biological activity was treated the same way. Triplicate samples of biodegradation and control were analyzed.

4.3 RESULTS AND DISCUSSION

4.3.1 Purification and Identification of Microcystin-LR

The raw water collected from the Ferintosh treatment facility contained a substantial amount of organic carbon, illustrated by HPLC chromatograph (Figure 4.4). The dissolved organic carbon was 26 mg/L. TOC values between 10 and 50 mg/L have been observed in lake water with substantial algae present (Hoyer et al. 1985). The majority of the organic matter in the methanol extract appears more polar (shorter retention time) than microcystin-LR by comparison of HPLC chromatograms a and b (Figure 4.4). The freeze dried fractions also contained a substantial quantity of inorganic residue. After the methanol extraction, approximately 40% of the microcystin-LR was recovered (Table 4.1). A definite peak of microcystin-

LR can not be identified in the HPLC chromatograph (Figure 4.4b), but the position of microcystin-LR in the profile was determined with the PP bioassay (Figure 4.4c).

Microcystin-LR in the Ferintosh raw water sample was visible after elution from the second gradient analysis (0.1% TFA-water/acetonitrile). The sample and microcystin-LR standard were almost chromatographically identical (Figure 4.5). The sample and microcystin-LR standard did not clearly co-migrate with the third gradient (Figure 4.6). This may have been caused by variations in the elution gradient between the standard and sample run. To avoid this problem, co-migration of the sample and standard microcystin-LR (of approximately the same concentration as the sample) was determined with an isocratic HPLC run, using 10 mM ammonium acetate:acetonitrile (76:24) as the mobile phase, thereby avoiding gradient mixing. The retention time of the standard and sample microcystin-LR were almost identical (Figure 4.7). A single peak was obtained when mixing the standard microcystin-LR and the Ferintosh sample essentially confirming the identity of the microcystin in the Ferintosh water (Figure 4.7c).

A less polar microcystin analogue was also present in the sample, eluting approximately 8 minutes later than the sample (Figure 4.5c). The identity of the analogue was not determined. The concentration of the analogue was approximately 5 ng/L (microcystin-LR equivalents) and would not contribute substantially to the microcystin levels measured. The presence of more than one microcystin analogue has been observed in an algal bloom (Namikoshi et al. 1992) and in several strains of *Microcystis aeruginosa*

(Watanabe et al. 1991). Good agreement for the sample concentration was found between the protein phosphatase bioassay and the HPLC U.V. detection at 240 nm (Table 4.1).

4.3.2 Ferintosh Treatment Removal Profiles

The Ferintosh treatment train and sample location is shown in Figure 4.2. The plant operates on a need basis for the community, and the plant is designed to serve a population of 173 people at 0.25 m^3/day. The plant flow is approximately 0.1 m^3/d. Alum is added at a dose of ~100 mg/L. The removal profiles for both sampling days are shown in Figures 4.8 and 4.9. The detention time of each unit process was not determined but an approximation of the detention times was made. The approximate time adjusted elution profiles are shown in Figures 4.10 and 4.11. The amount of microcystin-LR removed at each stage of the purification is summarized in Table 4.2.

Toxin removal was observed at every stage in the treatment train. The amount of microcystin-LR removed by each stage was approximated by comparing the daily average microcystin-LR concentration after each stage of treatment with the average raw water concentration (Table 4.2). The negative value for coagulation/sedimentation on August 25 is misleading if compared with Figures 4.8 and 4.10. Some removal by coaglation and sedimentation appears to have occurred but the high values obtained for some samples after coagulation/sedimentation increased the average above the raw water average.

The concentration of microcystin-LR entering the water treatment plant varied over the time course of the day but appeared to be a maximum in the morning and the evening on October 1. The cyanobacteria tend to sink lower in the water in the evening and may be closer to the intake for the treatment plant. The variation in concentration in the raw water may also be a reflection of the variability of toxin concentration in the algal biomass (Carmichael and Gorham 1981), or variations in the release of microcystin-LR from the algae over the day. This supports the conventional wisdom that treatment plant intakes should be placed below the photic zone, and raw water should be drawn during the day time if possible.

Conventional treatment, coagulation, sedimentation and filtration was observed to remove some microcystin-LR. The removal of microcystin-LR by these treatment processes has not been observed to be significant in previous experimental studies. Approximately half of the microcystin-LR entering the GAC was removed (Table 4.2). However, in figures 4.8 to 4.11, it appears that regardless of the GAC influent or raw water concentration, a constant amount of microcystin-LR is present in the GAC effluent throughout the day on both sampling dates. The GAC effluent was chlorinated and stored in a reservoir before distribution. There was no reduction observed in the tap water concentrations compared with the effluent of the GAC. Therefore, as reported by others, chlorination does not achieve any reduction of microcystin-LR (Wheeler et al.1942, Hoffman 1976, Himberg et al.1989).

The TOC removal at each stage of the treatment is shown in Figure 4.12. The water was observed to have a green tinge after all stages of treatment with decreasing intensity from raw to treated. As mentioned in the methods, precipitation of organic matter occurred after acidification of the samples. The precipitate was green in colour and could be easily dispersed by shaking the sample bottle. The homogenized samples gave reproducible TOC values. High TOC values, 25 to 30 mg/L, at every stage of the treatment were observed. Coagulation has been shown to remove the high molecular weight, easy to flocculate polyhydroxy aromatic compounds the most efficiently (Bruchet et al. 1990), and in Norwegian lake water, the source with the greatest amount of lower-molecular weight organics was shown to have the highest coagulant dosage demand and the lowest TOC removal (Vik. et al. 1985). The molecular weight distribution of dissolved organic carbon (DOC) from algal waters has been characterized as predominately in the 1000 to 10,000 dalton range (Tuschall and Brezonik 1980). The poor removal of TOC observed in the Ferintosh plant may be a reflection of the poorer removability of lower molecular weight organics.

4.3.3 Camrose Treatment Removal

The presence of microcystin in the raw and treated water from the Camrose facility is depicted in Figure 4.13. The Camrose facility consists of PAC application (30 mg/L) with conventional processes and the treatment train is outlined in Figure 4.3. As with the Ferintosh data, microcystin is not completely removed from the drinking water. The finished water had an approximate microcystin concentration of 0.1 to 0.2 μg/L (microcystin-LR equivalents) on every sampling date despite variations in the raw water concentration. These data show that PAC and conventional treatment processes achieved some removal of microcystin-LR.

4.3.4 Sorption Isotherms

The equilibrium time for the sorption isotherms was determined to be one day with both pre-loaded and virgin carbon. Virgin and preloaded carbon sorption isotherms with laboratory grade water, and competitive sorption isotherms with lake water were determined for pure Microcystin-LR (Figures 4.14 to 4.16). The most distinguishing feature of the isotherms is the nonlinearity over the equilibrium concentration range in the competitive and pre-loaded isotherms. Non-linear isotherms have been observed with geosmin and 2-methylisoborneol (MIB), over a similar concentration range in both virgin and pre-loaded isotherms (Lalezary et al. 1986).

The data was divided into two distinct regions and the Freundlich parameters were calculated independently for each region of data. The Freundlich parameters are presented in Table 4.3 as above or below the point of intersection of the two isotherms, termed the break point. The initial concentration in the isotherms ranged from ~1μg/L to ~10 μg/L microcystin-LR. A general trend of decreasing final concentration with decreasing initial concentration was observed.

The non-linearity of the competitive and pre-loaded isotherms indicates the possibility that different removal mechanisms may predominate below the break point concentration. The break point in the isotherms occurs at approximately the same equilibrium concentrations in both competitive and pre-loaded experiments, ~0.15 μg/L microcystin-LR (Table 4.3). In contrast, the virgin carbon isotherm does not appear to have a break in linearity. This suggests that the change in mechanism is related to the concentration of microcystin-LR, the presence of algal natural organic matter (NOM) and the biofilm. The Freundlich constants change dramatically after the break point. The constants do not correspond with expected values, i.e. the very large capacity predicted for the carbon. The implication of the observed steep slope in the isotherms is that below concentrations of ~0.15 μg/L microcystin-LR, very little microcystin-LR will be removed from solution by activated carbon. This may explain the constant residual concentration of microcystin observed in the Ferintosh and Camrose drinking water.

The Freundlich capacity (Kf) of the carbon was observed to decrease significantly in the competitive isotherm compared with the virgin isotherm (Table 4.3) (Figures 4.14 and 4.15). The presence of NOM in the water has been shown to cause a reduction in the capacity of activated carbon for a number of target compounds (Najm et al. 1991, Qi et al. 1992). The reduction in capacity was found to be dependant on the nature of the organic matrix in the water (Herzing et al. 1977). Therefore the competitive effect of natural organic matter on microcystin-LR sorption was evaluated by conducting the isotherms with water removed from the Ferintosh treatment train just prior to entering the GAC bed.

Microcystin-LR has a molecular weight of 994 and the the molecular weight distribution of dissolved organic carbon (DOC) from algal waters is predominately in the 1000 to 10,000 dalton range (Tuschall and Brezonik 1980). The majority of NOM in the water matrix was more polar than microcystin-LR and it had considerable absorbance at 240 nm (Figure 4.4a), suggesting that the organics could be proteinaceous in nature (Campbell and Dwek 1984). Therefore the NOM probably has similar adsorption characteristics on activated carbon and competes readily with microcystin-

LR for adsorption sites. The TOC of the water matrix in the competitive isotherms was very high, 17 mg/L, compared to the concentration of microcystin-LR (μg/L). This large difference in concentration was probably the main factor for the observed decrease in capacity in the competitive isotherms. A very minor factor contributing to the reduction in the capacity of microcystin-LR could be the presence of the microcystin analogue in the water matrix used for the competitive sorption isotherms (Figure 4.5c). The analogue was less polar than microcystin-LR and present at approximately 5 to 7 ng/L. However, the presence of several analogues in the raw water is very likely (Namikoshi et al. 1992) and may be a factor in decreasing activated carbon capacities for microcystin toxin in other treatment situations. The mutual interference of structurally similar analogues was studied with p-nitrophenol and phenol (Fritz et al. 1980). The data indicated that the adsorption of the more weakly adsorbing compound was affected more by the presence of a structural analogue.

The equilibrium isotherm has also been found to be very dependent on initial isotherm concentration of the target compound because of the competition with NOM (Najm et al. 1991). Initial concentrations of, ~1 to ~10 μg/L microcystin-LR, covering the range of concentration observed at the Ferintosh treatment faciltity, were used in the isotherms. Therefore the reduction in the capacity observed in the competitive adsorption isotherm can be attributed to competition between microcystin-LR and algal NOM.

The Freundlich capacity (K_f) of the pre-loaded carbon isotherm was observed to decrease significantly compared with the virgin isotherm (Table 4.3) (Figures 4.14 and 4.16). The pre-loading of NOM on activated carbon has been shown to cause reductions in the capacity of carbon for target compounds (Carter et al. 1992, Andrews et al. 1990). The biofilm in GAC filters has been shown to be more prevalent in the top of the GAC filter (Hascoet et al. 1986). To evaluate the decrease in carbon capacity from pre-loading and the presence of a biofilm the carbon samples used in the pre-loaded experiments were removed from the top of the GAC bed at Ferintosh. The activated carbon collected from the Ferintosh treatment facility had been in the GAC bed for approximately five months. Scanning electron microscope photographs of virgin and uncrushed Ferintosh carbon depict the difference in the surface of virgin and pre-loaded activated carbon (Figure 4.19).

The isotherms conducted with crushed and uncrushed pre-loaded carbon were not the same (Figure 4.18). Both the crushed and uncrushed isotherms were non-linear over the equilibrium concentration range. The break point also occurred at a similar concentration. However, a decrease in the capacity of the carbon was only observed with the uncrushed carbon. Crushing pre-loaded carbon has been suggested to cause an opening of pores that may be blocked by the pre-loaded organic matter resulting in exposure of virgin carbon surfaces (Carter et al. 1992). The virgin surfaces created presumably cause an increase in the capacity of the pre-loaded carbon. Therefore the uncrushed carbon isotherm is likely more indicative of the effects of pre-loading on microcystin-LR removal by activated carbon.

Biodegradation of microcystin-LR (measured as a decrease in the equilibrium microcystin-LR concentration with time) by the biofilm on the Ferintosh carbon was not observed over the course of one month (data not shown). Therefore some microcystin-LR previously sorbed onto the carbon from the Ferintosh GAC bed would presumably be present on the pre-loaded carbon. Attempts to desorb microcystin toxin from carbon have been unsuccessful (Stewart et al. 1950, Falconer et al. 1989) and were not tried in our experiments. The presence of pre-loaded microcystin-LR on the carbon would cause a decrease in the capacity of the pre-loaded carbon.

The slope (1/n) of the microcystin-LR isotherms decreases from the virgin to the pre-loaded and competitive isotherms above the break point (Table 4.2). The organic matter present in algal waters has been described as being high in protein and carbohydrate and resistant to microbial degradation (Scully et al. 1988, Fedorak and Huck 1988, and Hoyer et al. 1985). Proteins generally represent the largest single fraction of organic components of many algae (Scully et al. 1988), and 30% of the extracellular dissolved organic matter produced by *Microcystis aeruginosa* was observed to be amino acids (Jensen 1985). The extracellular matrix of bacteria also consists of protein and carbohydrates (Alberts et al. 1989). Therefore with both pre-loading by algal NOM and the presence of a biofilm, the surface of the activated carbon conceivably changes from a non-polar surface to a more polar surface. The change to a more polar carbon surface may cause a change in the removal mechanism from adsorption for the virgin carbon to partitioning into a surface organic layer for the pre-loaded carbon. Microcystin-LR, being a cyclic peptide, likely has an affinity for the more organic layer. The slope of the isotherm has been suggested to be indicative of the heterogeneity in adsorption site energy (Carter et al. 1992). Increases in the slope were interpreted as a decrease in the heterogeneity of adsorption site energies. The decrease in the slope observed with microcystin-LR could be due to a change in the sorption mechanism and/or a greater range of sorption site energies.

Isotherm data determined with Calgon F-100 carbon and Norit 0.8 Supra carbon are shown on the virgin and competitive isotherms (Figures 4.14 and 4.15). These two carbon types were found to be the most effective at adsorbing microcystin in a column test and a pilot plant study (Falconer et al. 1983 and 1989). Although limited data was obtained for both carbon types, it appears that they have a greater capacity for microcystin-LR in the virgin carbon isotherm (Figure 4.14). In contrast, the capacity of both Calgon F-100 and Norit 0.8 Supra carbon appears the same as Haycarb carbon in the competitive isotherm (Figure 4.15). This suggests that the removal of microcystin-LR by activated carbon will be essentially the same with any of the three carbon types in treatment plants.

A decrease in the rate of sorption of microcystin-LR with the pre-loaded carbon compared to virgin carbon probably occurs because of blockage of the micropores by the bio-film (Figure 4.19). A decrease in the apparent phenol adsorption rate for activated carbon colonized with bacteria has been observed (Kindzierski et al. 1992). Therefore longer contact times may result in an increase the capacity of pre-loaded GAC for microcystin.

In comparison of the pre-loaded and competitive isotherms (Figure 4.17), the 95% confidence intervals for the predicted load overlap suggesting there is not a significant difference in the two isotherms. This implies that virgin carbon in the presence of NOM will not perform that much differently than pre-loaded carbon in GAC beds. Therefore changing the carbon bed from pre-loaded carbon to virgin carbon would not necessarily result in better removal of microcystin-LR.

4.3.5 Implications of Treatment Findings

This study has demonstrated that the water treatment processes used in this study, including activated carbon, left a readily detectable residue of microcystin-LR in the final product. This implies that water consumers may be chronically exposed to microcystin-LR for the duration of the cyanobacteria bloom months. For both Ferintosh and Camrose the residual concentration of microcystin-LR was approximately 0.1 to 0.5 μg/L. The cyanobacteria blooms of 1992 were not as intense as had been observed in 1990 or 1991, and the data was also collected in the fall when the cyanobacteria blooms were not as large compared with summer months. Therefore, the concentrations observed in the finished water may be lower than during intense cyanobacteria blooms. In the derivation for maximum acceptable concentrations for drinking water guidelines, it is assumed that an individual consumes 1.5 L of water per day (Guidelines for Canadian Drinking Water Quality 1989). At this level of consumption an individual would have consumed approximately 0.1 to 0.8 μg of microcystin-LR per day. There was no community wide incidence of acute illness reported in Ferintosh or Camrose during the course of the study. These data suggest that previously reported incidents of acute human illness related to cyanobacteria toxins in drinking water likely occurred at much higher concentrations of toxin in the water. This is quite probable in that three human incidents involved dosing a cyanobacteria bloom in the raw water with copper sulphate (Tisdale 1930, Falconer et al. 1983a, and Bourke et al. 1983) presumably resulting in lysis of the cyanobacteria cells and release of toxin into the water (Kenefick et al. 1993).

In specifying the maximum acceptable concentration for drinking water guidelines, the concentration must be achievable with drinking water treatment processes and measurable by available analytical methods (Guidelines for Canadian Drinking Water Quality 1989). The data suggest that concentrations in the vicinity of 0.1to 0.5 μg/L microcystin-LR are achievable by drinking water treatments, measurable by the PP bioassay. Although there was no readily apparent contribution to acute illness among consumers, the chronic health risks that may be associated with microcystin-LR consumption deserve further attention.

4.4 SUMMARY AND CONCLUSIONS

The PP bioassay was applied to measure microcystin-LR at the trace concentrations found in drinking water sources. Reproducible results were obtained with both treated and raw water. Therefore the PP bioassay is a useful analytical technique for monitoring microcystin at environmentally relevant concentrations.

This study confirmed the presence of microcystin-LR in the drinking water. Microcystin-LR in the water sample was identified by comparison of its chromatographic properties with standard microcystin-LR.

The competitve effects of NOM, and pre-loading on activated carbon were evaluated. Pre-loading and competitive NOM were observed to cause a reduction in the capacity of activated carbon for microcystin-LR. The pre-loaded and competitive adsorption isotherms were not significantly different. The most distinguishing feature of the isotherms was the non-linearity over the equilibrium concentration range. The isotherms suggest that microcystin-LR will not be removed by GAC much below ~0.1 μg/L microcystin-LR.

Analysis of a full-scale treatment facility indicated that conventional treatment processes remove some microcystin-LR from solution. GAC filtration and PAC with conventional treatment in full-scale application were observed to remove microcystin-LR from solution . In both treatment facilities, a residual concentration of microcystin-LR was observed. This finding is consistent with the isotherm results and implies that consumers will be exposed to chronic levels of microcystin-LR when the toxin is present in the raw water.

Table 4.1 Quantitation of Microcystin-LR Purification from Drinking Water.

Sample	Fraction	Amount of microcystin -LR (ng) PP bioassay	Amount of microcystin -LR (ng) HPLC/UV
Initial Sample		410	ND
Methanol Extraction	pool of 4 extractions	160	ND
1st Ammonium Acetate/Acetonitrile	collected peak fractions	166	ND
0.1% TFA/ acetonitrile	56 min.	82	93
2nd Ammonium Acetate/Acetonitrile	27 min. 28 min.	50 35	125

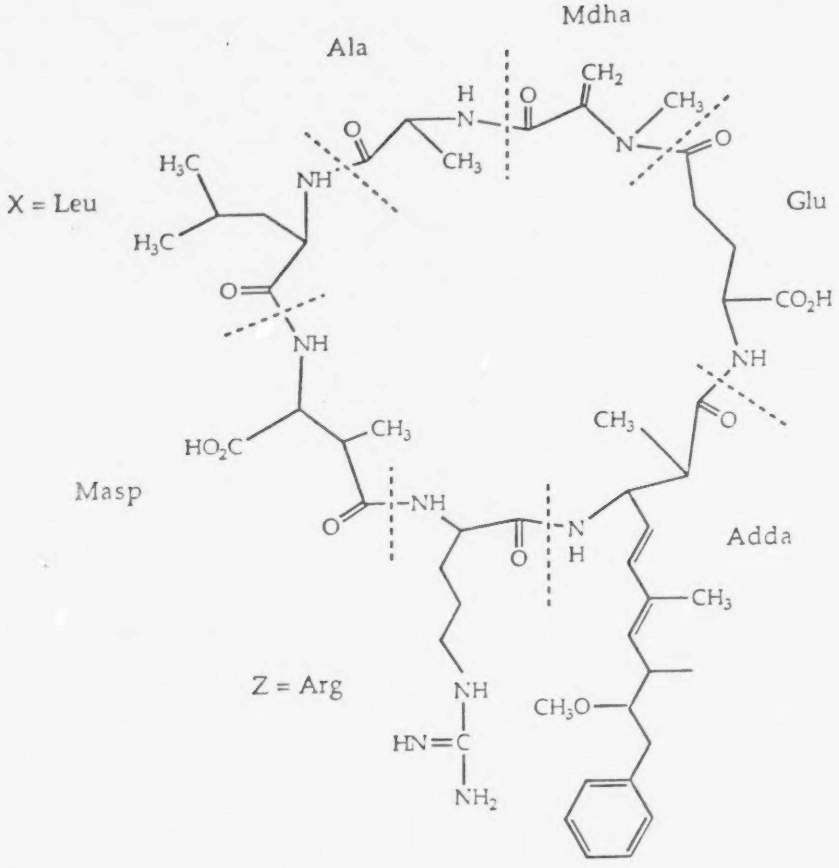
N.D. = not detectable

Table 4.2. Removal of microcystin-LR at each stage of the Ferintosh treatment train.

Unit Process	August 25			October 1		
	Average Concentration (µg/L)	Cumulative % Removal from Raw	% Removal of Influent to Process Step	Average Concentration (µg/L)	Cumulative % Removal from Raw	% Removal of Influent to Process Step
Raw	2.92 ± 0.80	-	-	1.15 ± 0.11	-	
Coag/Sed	3.08 ± 1.13	- 5.6	0	0.70 ± 0.05	39.0	39.1
Filtration	1.23 ± 0.23	58.0	60.1	0.60 ± 0.07	47.7	14.3
GAC bed	0.49 ± 0.09	83.1	60.2	0.34 ± 0.05	70.5	43.3
Chlorination	0.53 ± 0.19	82.0	0	0.34 ± 0.06	70.6	0

Table 4.3. Comparison of Freundlich Isotherm Parameters

Isotherm	**Break Point (C_{BP})**	**$C_f > C_{BP}$**		**$C_f < C_{BP}$**	
	µg/L microcystin-LR	**K_F**	**1/n**	**K_F**	**1/n**
Virgin	-	50.12	1.04	50.12	1.04
Competitive	0.15	12.77	0.53	188	2.05
Pre-Loaded	0.15	6.22	0.58	854	3.03



Mdha
Ala
X = Leu
Glu
Masp
Adda
Z = Arg
H
O
CH$_2$
N
CH$_3$
NH
H$_3$C
H$_3$C
CO$_2$H
HO$_2$C
CH$_3$O
HN=C
NH$_2$

Figure 4.1 Structure of Microcystin-LR.

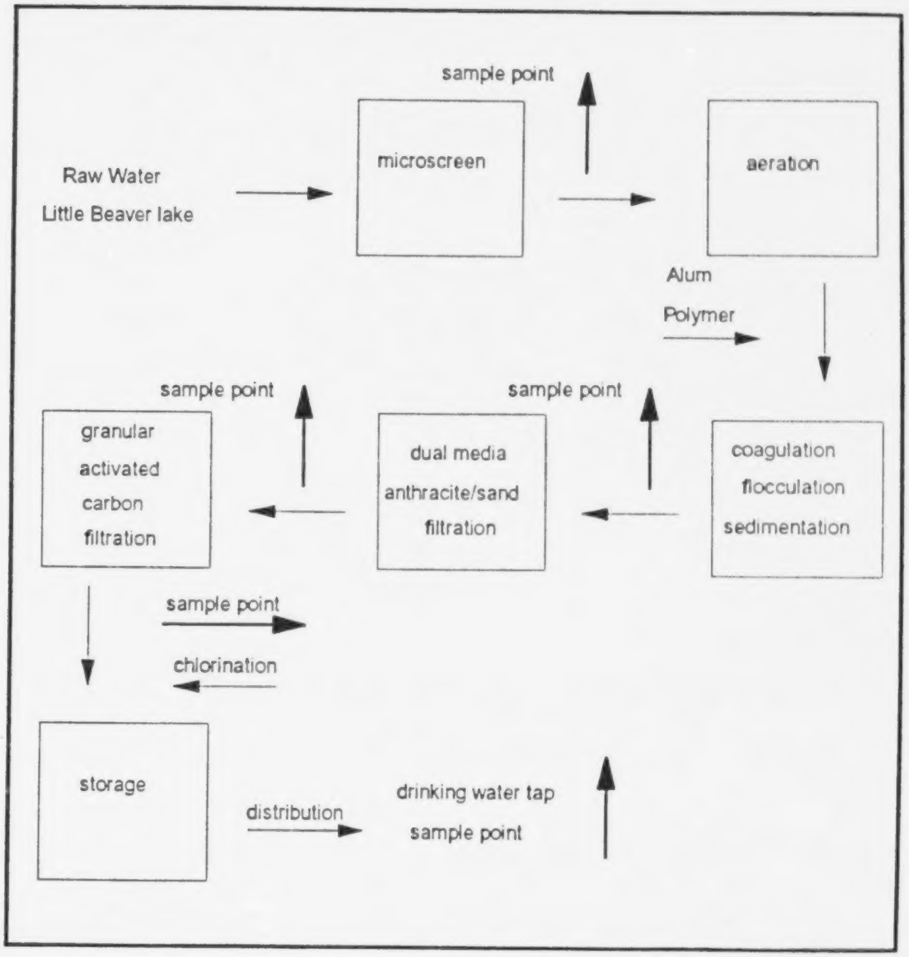

sample point
Raw Water
Little Beaver lake
microscreen
aeration
Alum
Polymer
sample point
sample point
granular
activated
carbon
filtration
dual media
anthracite/sand
filtration
coagulation
flocculation
sedimentation
sample point
chlorination
storage
distribution
drinking water tap
sample point

Figure 4.2 Flow Diagram of the Ferintosh Treatment Train.

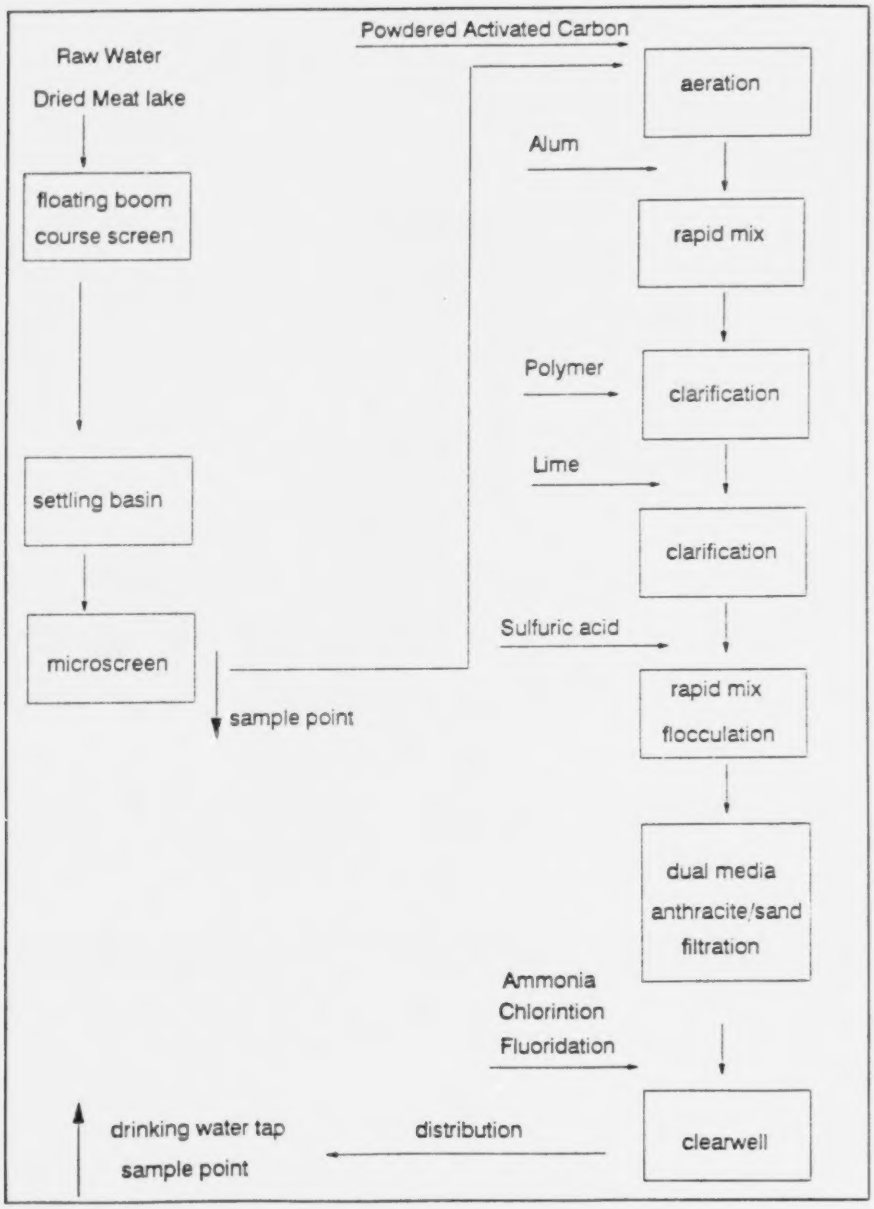

Raw Water
Dried Meat lake
floating boom
course screen
settling basin
microscreen
sample point
Powdered Activated Carbon
aeration
Alum
rapid mix
Polymer
clarification
Lime
clarification
Sulfuric acid
rapid mix
flocculation
dual media
anthracite/sand
filtration
Ammonia
Chlorintion
Fluoridation
clearwell
distribution
drinking water tap
sample point

Figure 4.3 Flow Diagram of the Camrose Water Treatment Train.

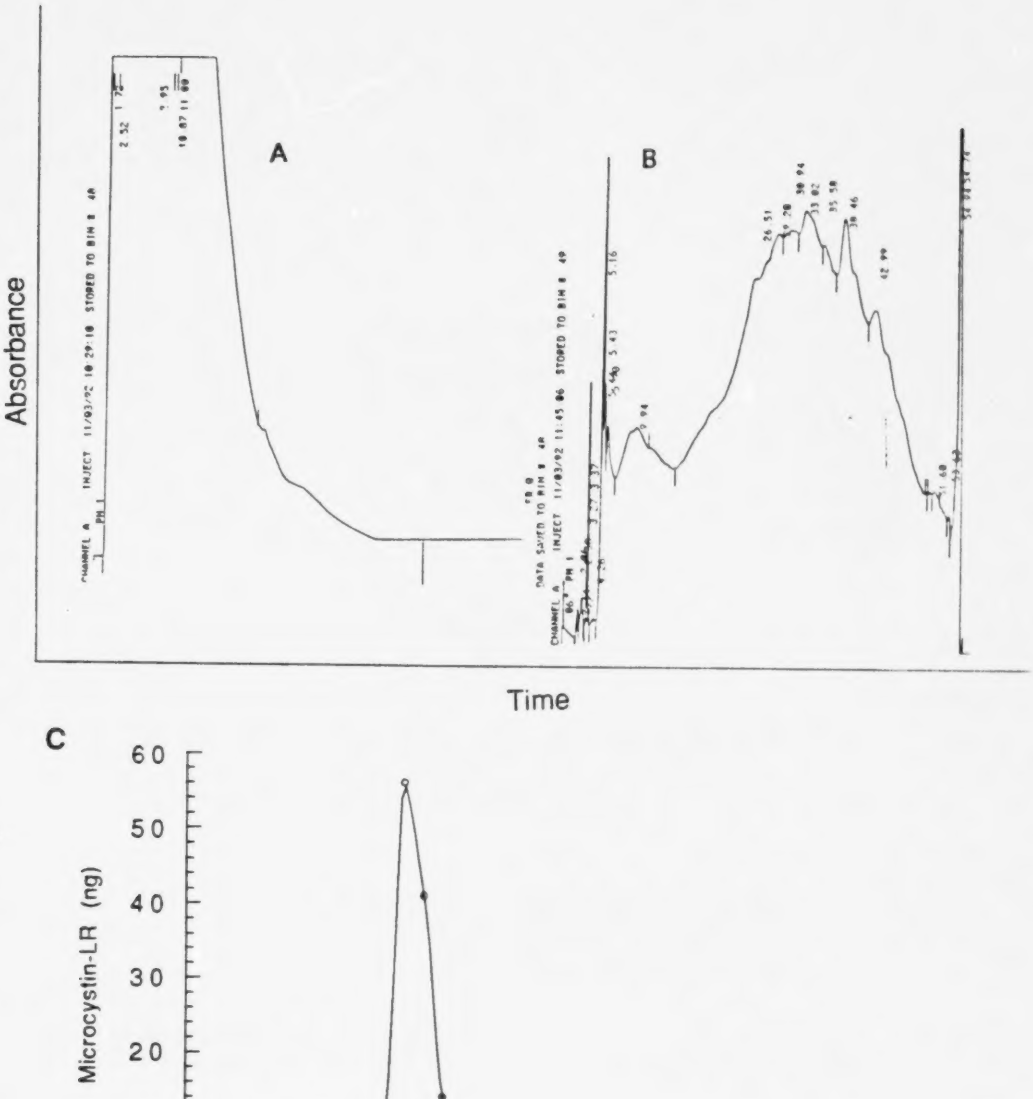


Figure 4.4 Purification of Microcystin-LR from Background Organic Matter: Elution with Ammonium Acetate/Acetonitrile, pH 6.5.
A) Elution profile of background organic matter. absorbace at wavelength 240 nm B) Elution profile of microcystin-LR sample, absorbace at wavelength 240 nm. C) PP bioassay profile of chromatograph B.

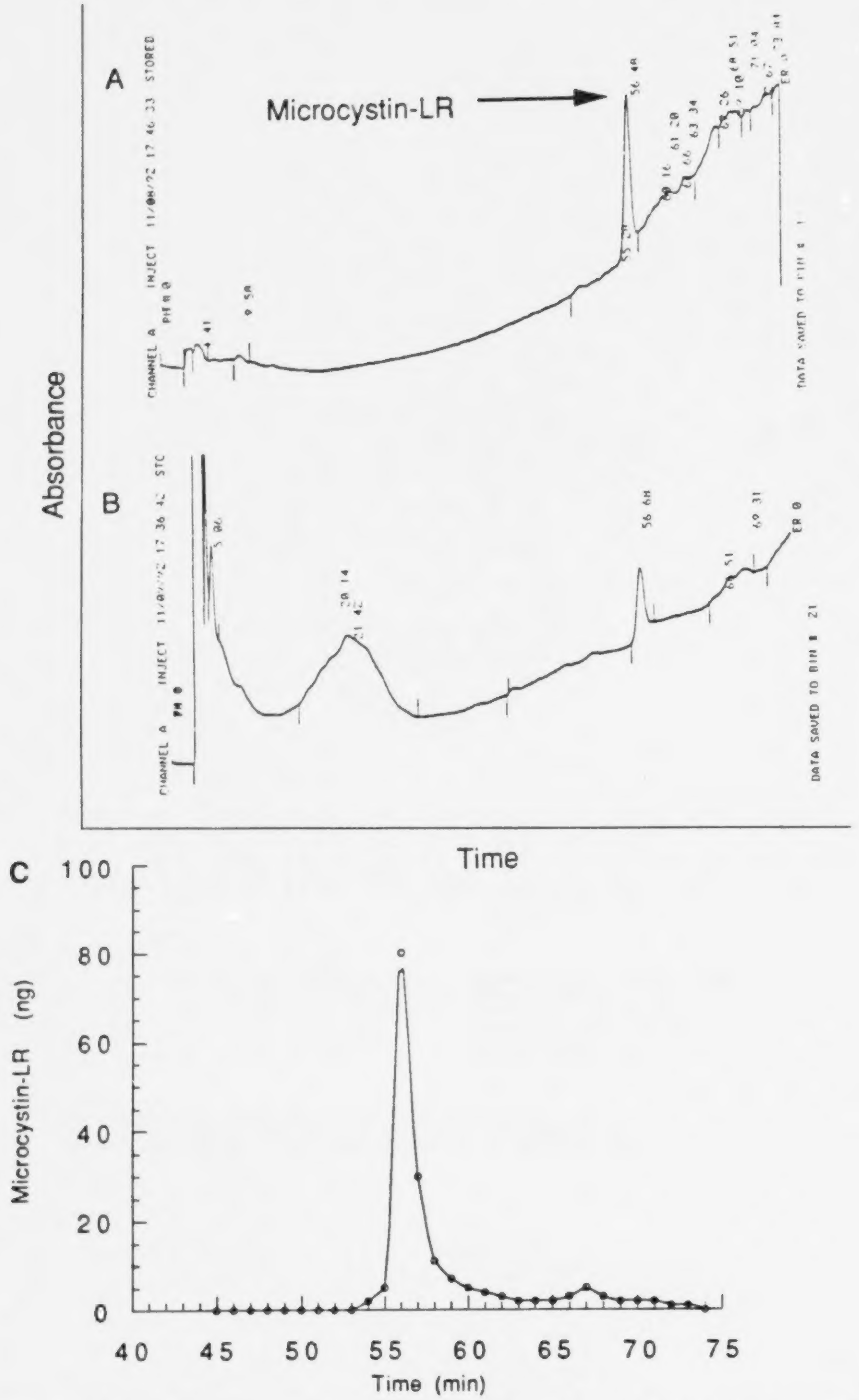


Figure 4.5 Identification of Microcystin-LR in the Drinking water: Elution with 0.1% TFA in Water/Acetonitrile, pH 2.0.
A) Standard microcystin-LR (200 ng), absorbace at wavelength 240 nm. B) Ferintosh drinking water sample, absorbace at wavelength 240 nm C) PP bioassay profile of chromatograph B.

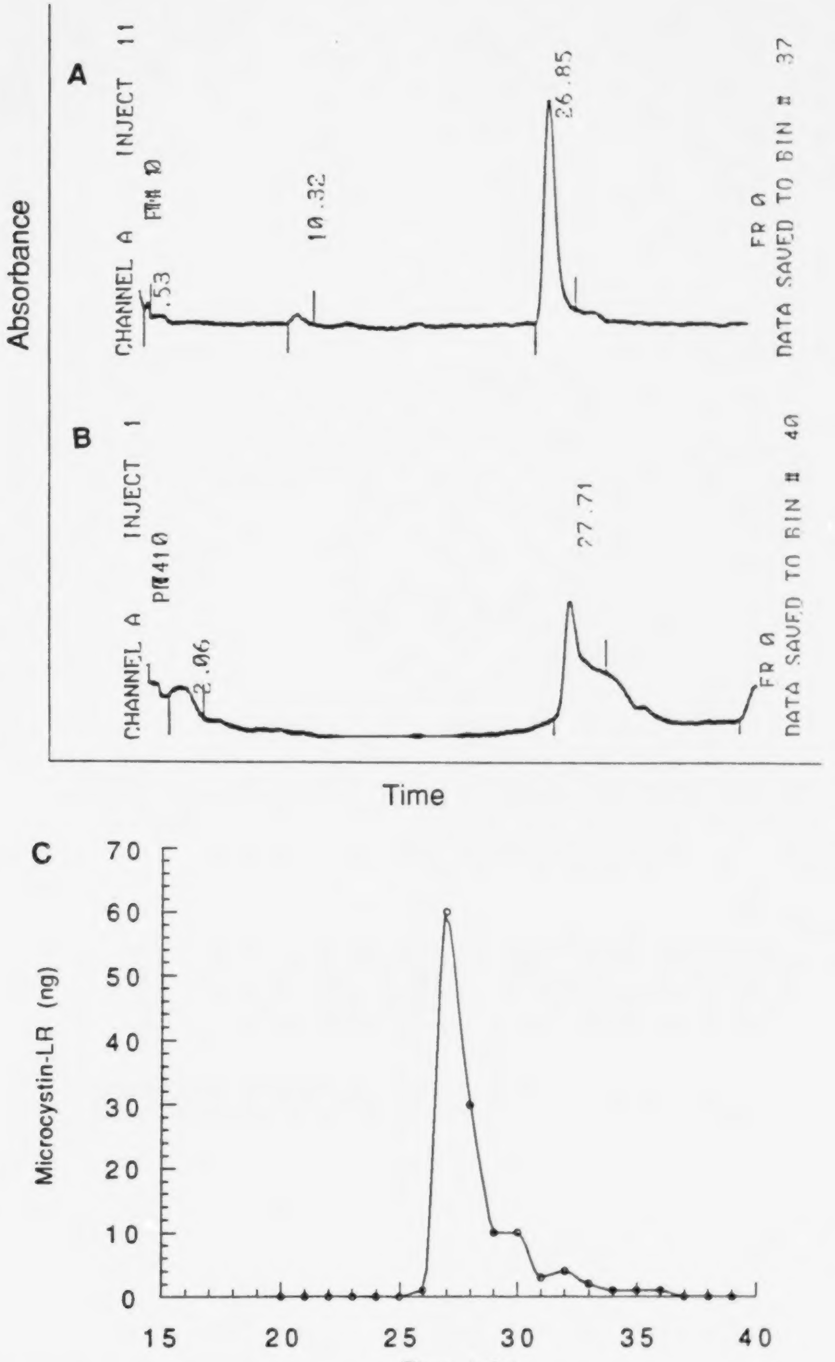


Figure 4.6 Identification of Microcystin-LR: Elution with Ammonium Acetate/Acetonitrile, pH 6.5.

A) Standard microcystin-LR (200 ng),absorbace at wavelength 240 nm. B) Ferintosh drinking water sample, absorbace at wavelength 240 nm. C) PP bioassay profile of chromatograph B.

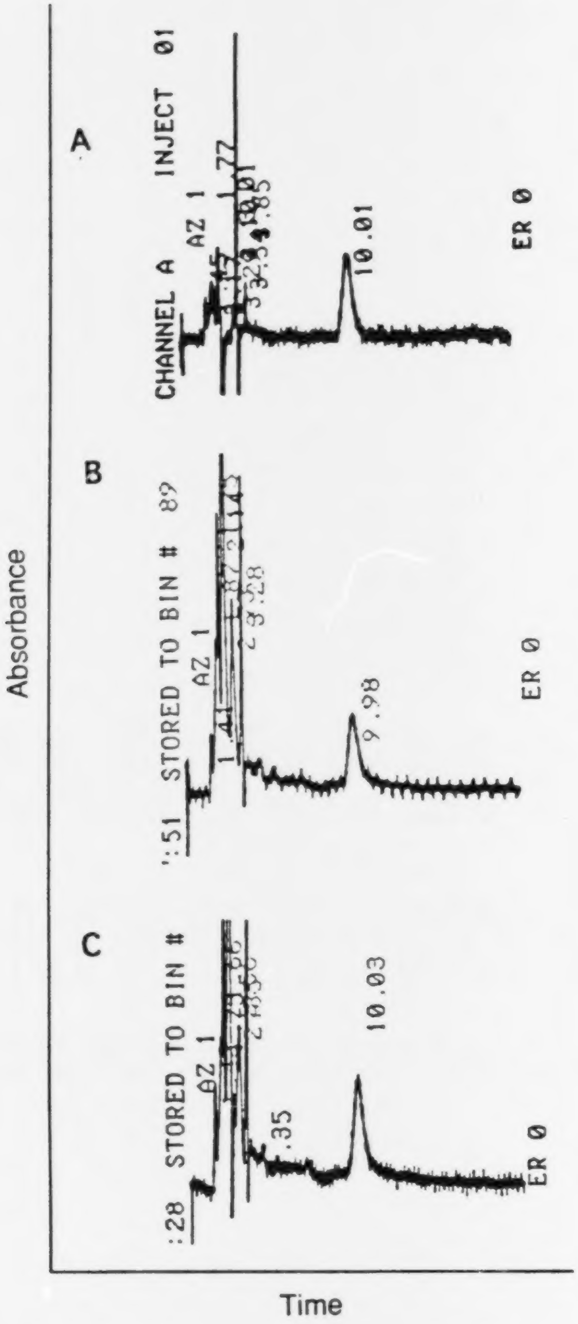


Figure 4.7 Isocratic Identification of Microcystin-LR: Elution with Ammonium Acetate/ Acetonitrile, pH 6.5, absorbace at wavelength 240 nm. A) Standard microcystin-LR (50 ng). B) Ferintosh drinking water sample. C) Mixture (50:50) of standard microcystin-LR and Ferintosh drinking water sample.

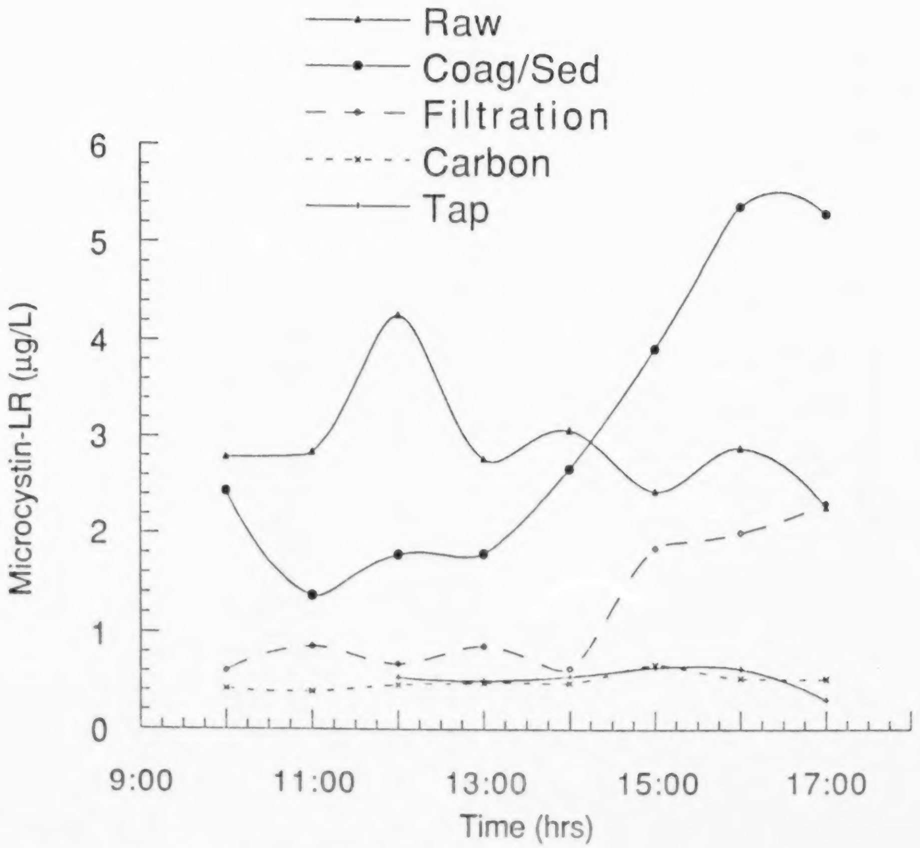


Figure 4.8 Microcystin-LR Concentration Profile at the Ferintosh Treatment Facility, August 25 1992.

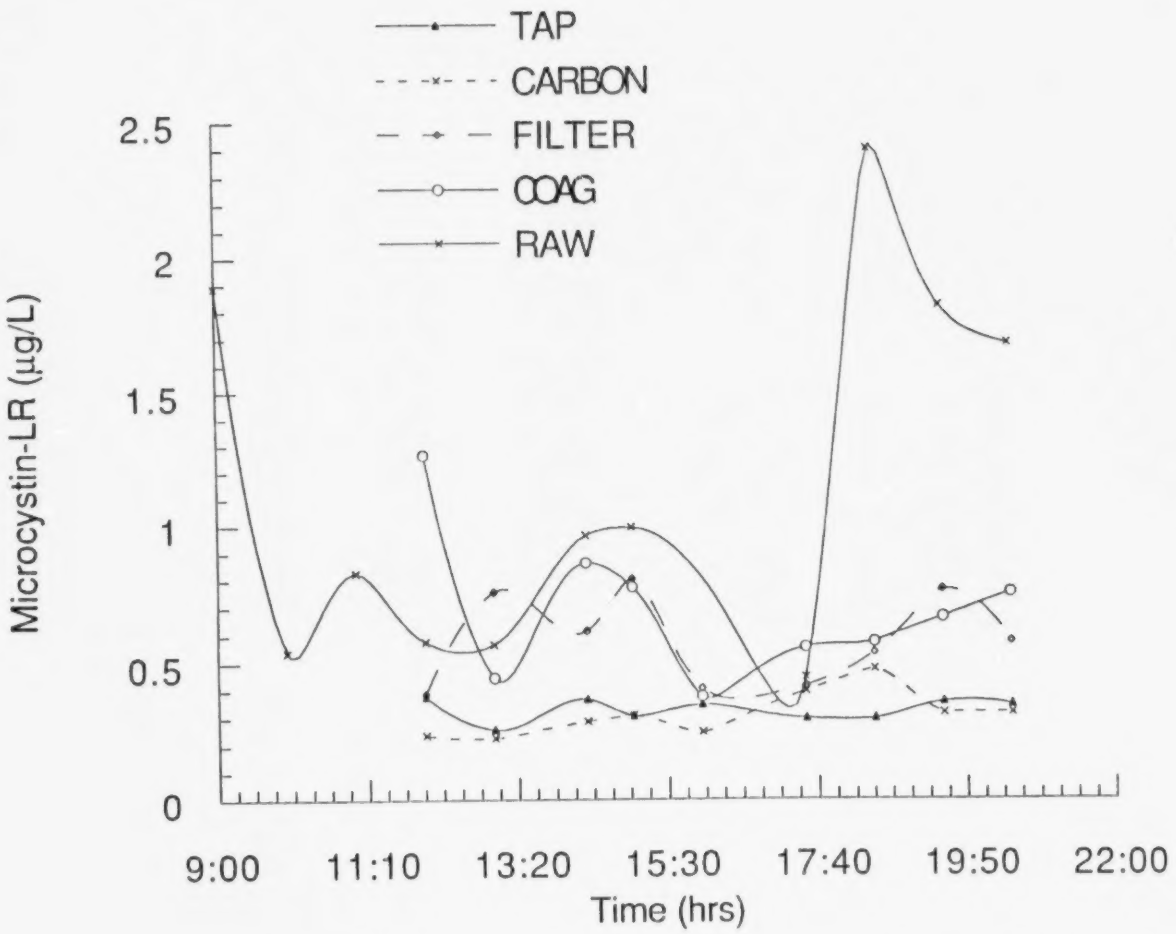


Figure 4.9 Microcystin-LR Concentration Profile at the Ferintosh Treatment Facility, October 1 1992.

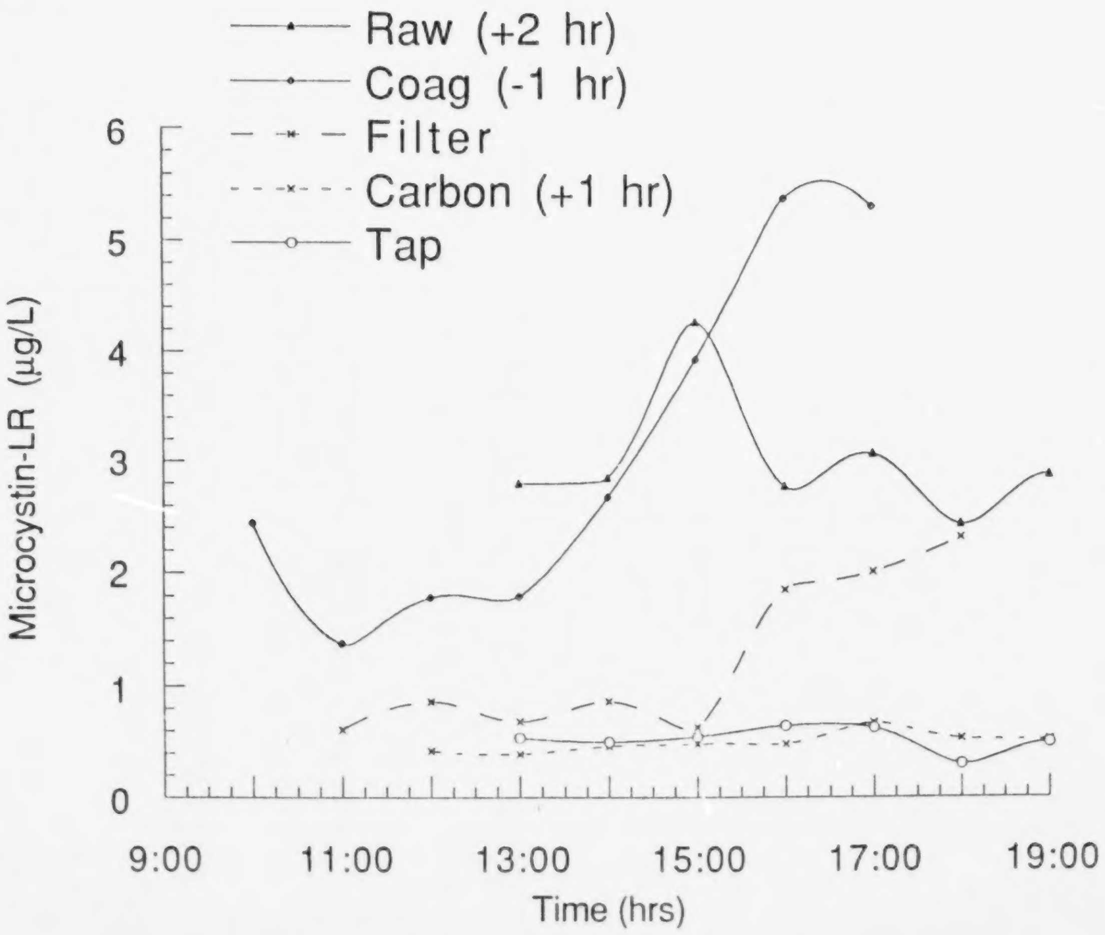


Figure 4.10 Time Adjusted Concentration Profile of Microcystin-LR, August 25 1992.

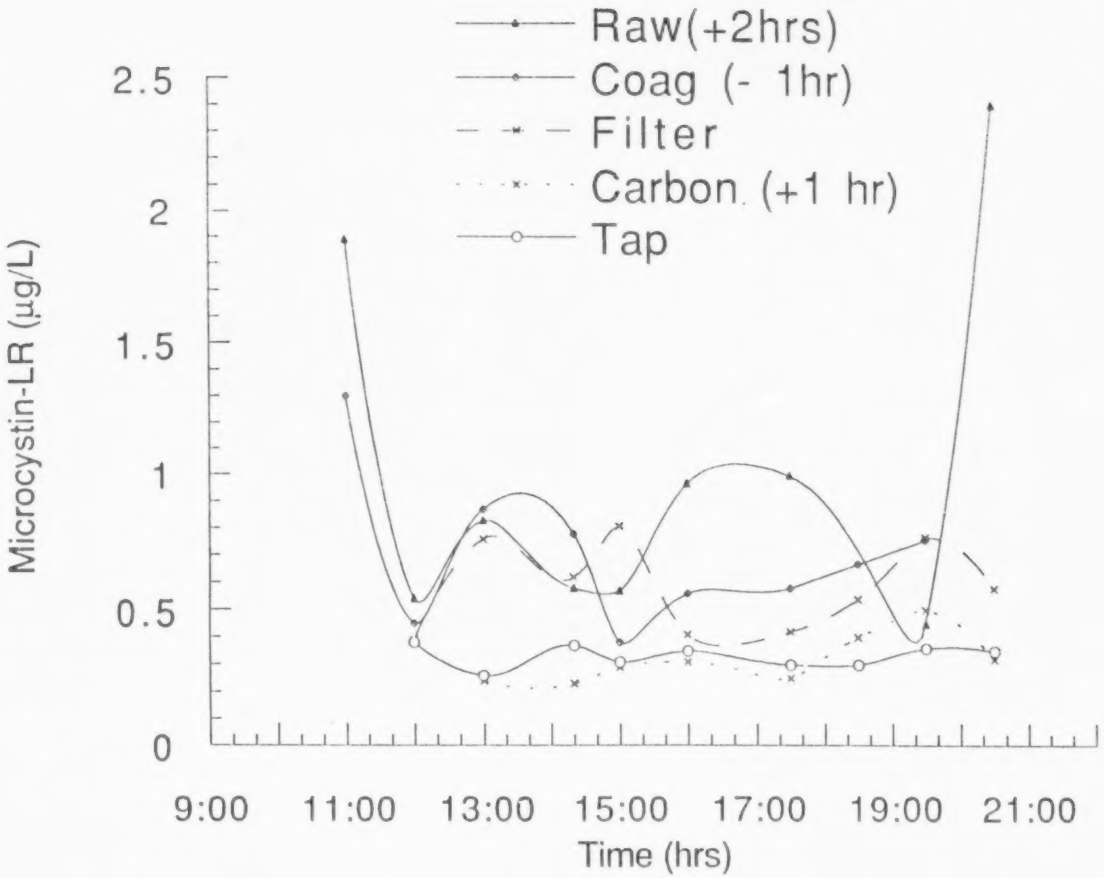


Figure 4.11 Time Adjusted Concentration Profile of Microcystin-LR, Ferintosh , October 1 1992.

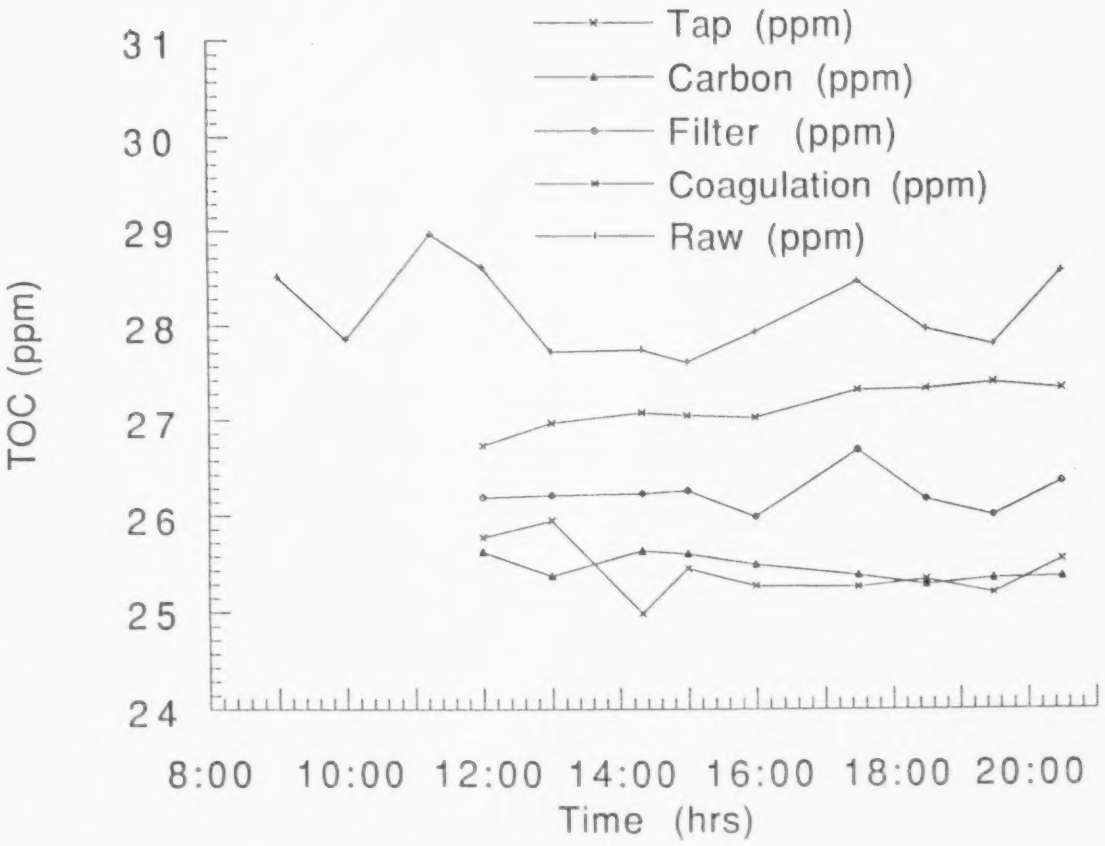


Figure 4.12 Total Organic Carbon (TOC) Concentration Profile, Ferintosh October 1 1992.

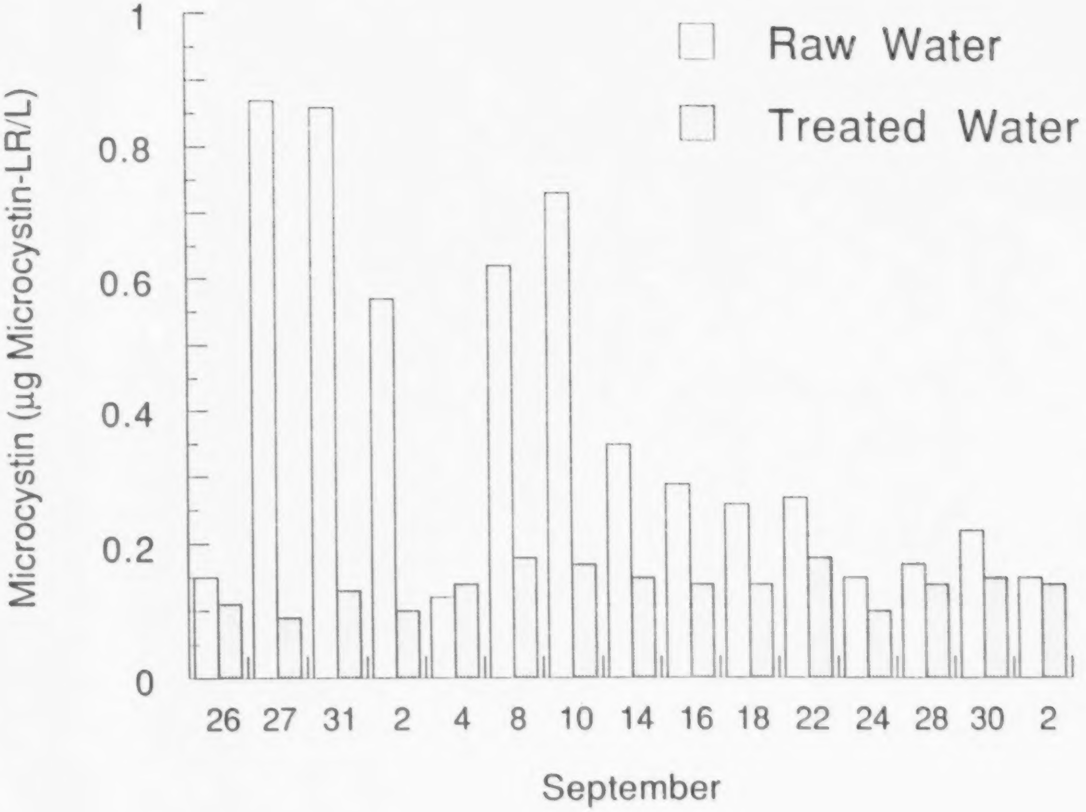


Figure 4.13 Camrose Raw and Treated Total Microcystin Concentrations, September 1992.

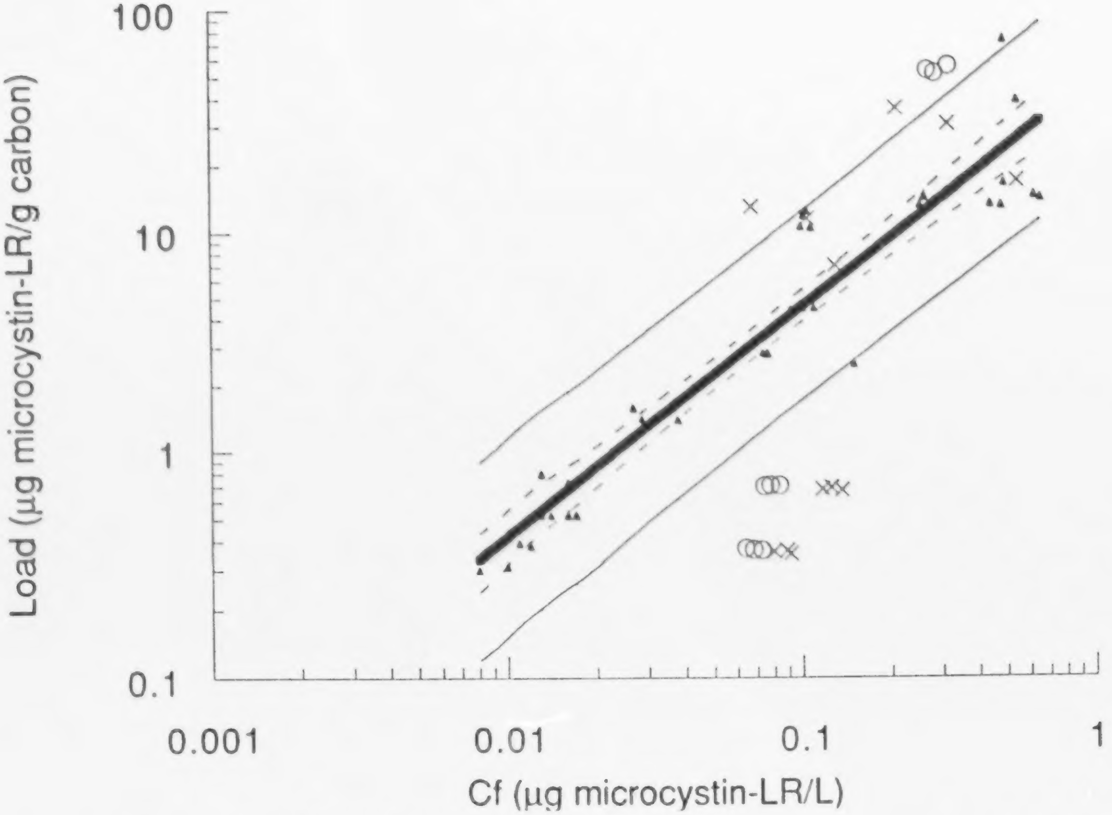


Figure 4.14 Virgin Carbon Sorption Isotherm for Microcystin-LR.
The virgin carbon isotherm was determined in lab grade water, pH 7.8, with virgin Haycarb carbon, indicated by Δ. The 95% confidence intervals for the mean load (dashed line) and predicted load (solid line) are shown. For comparison, data points determined with Calgon F-100 carbon, indicated by "o", and Norit 0.8 Supra carbon, indicated by "x" are shown.

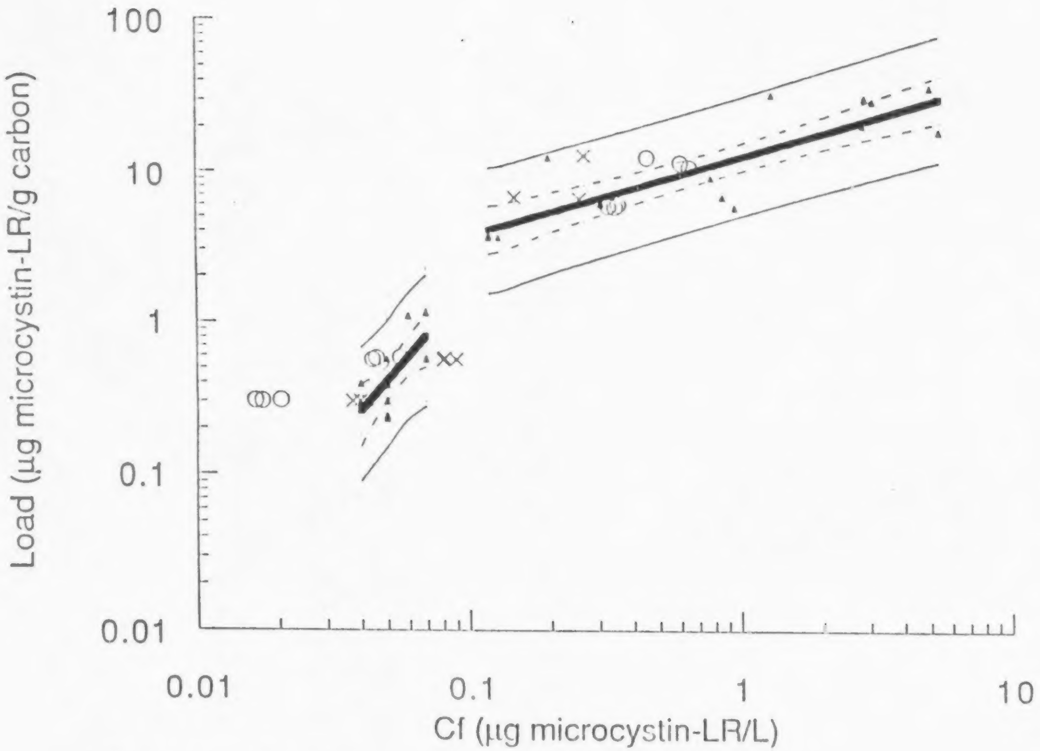


Figure 4.15 Competitive Sorption Isotherm for Microcystin-LR.
The competitve isotherm was determined in water removed from the Ferintosh treatment train prior to the GAC bed. The TOC of the water was 17 mg/L and the pH was 7.8. The isotherm was determined with virgin Haycarb carbon, indicated by Δ. The 95% confidence intervals for the mean load (dashed line) and predicted load (solid line) are shown. For comparison, data points determined with Calgon F-100 carbon, indicated by "o", and Norit 0.8 Supra carbon, indicated by "x", are shown.

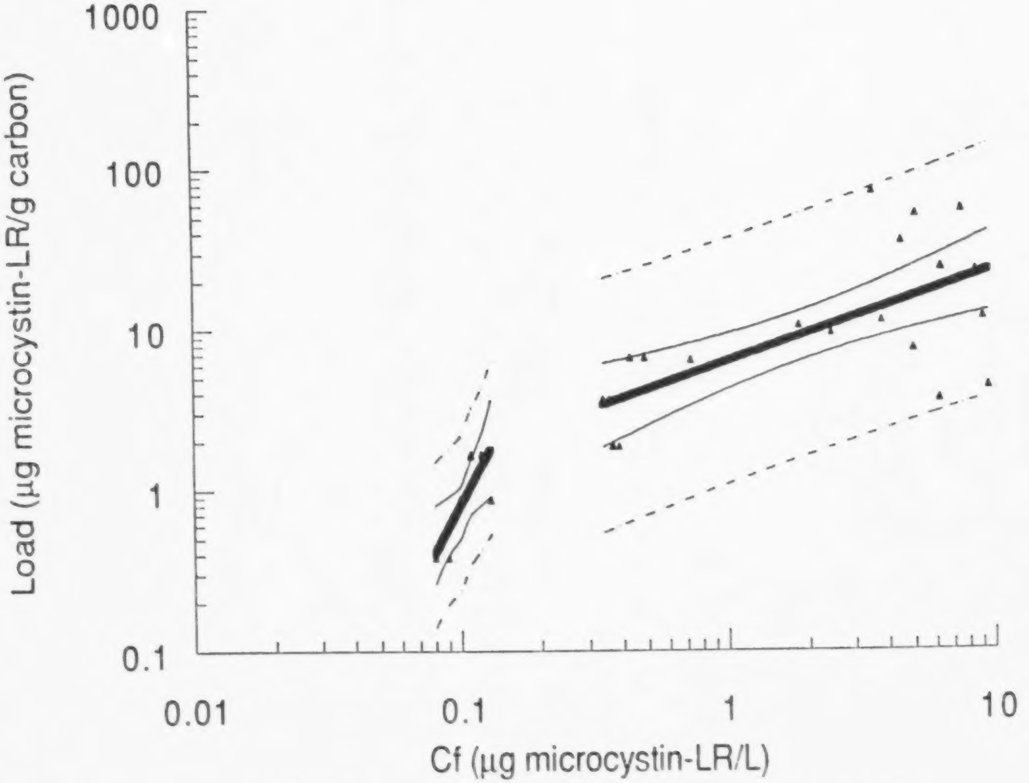


Figure 4.16 Pre-loaded Sorption Isotherm for Microcystin-LR.
The pre-loaded carbon isotherm was determined in lab grade water, pH 7.8, with Haycarb carbon removed from the Ferintosh GAC bed. The carbon was pre-loaded for 5 months. The 95% confidence intervals for the mean load (dashed line) and predicted load (solid line) are shown.

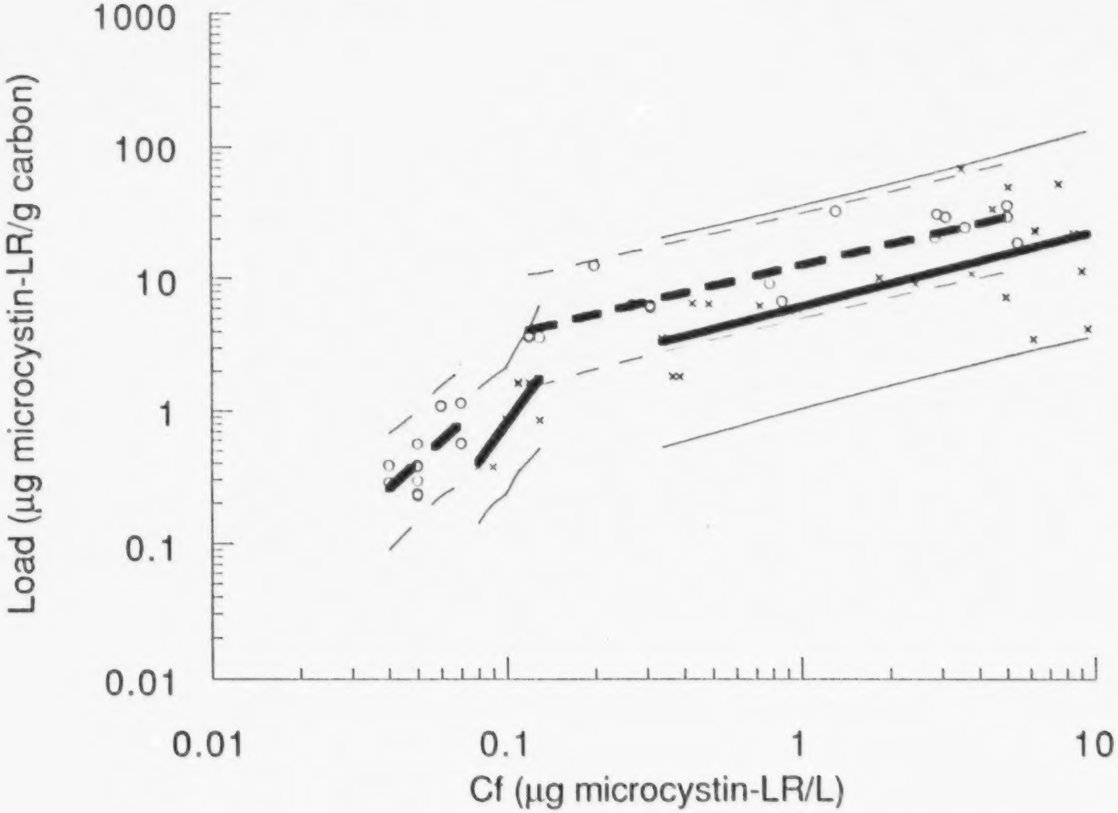


Figure 4.17 Comparison of competitive and pre-loaded isotherms.
The competitve isotherm is indicated by dashed lines and the sample points by "o". The pre-loaded isotherm is indicated by solid lines and the sample points by "x". The 95% confidence intervals for the predicted load are shown.

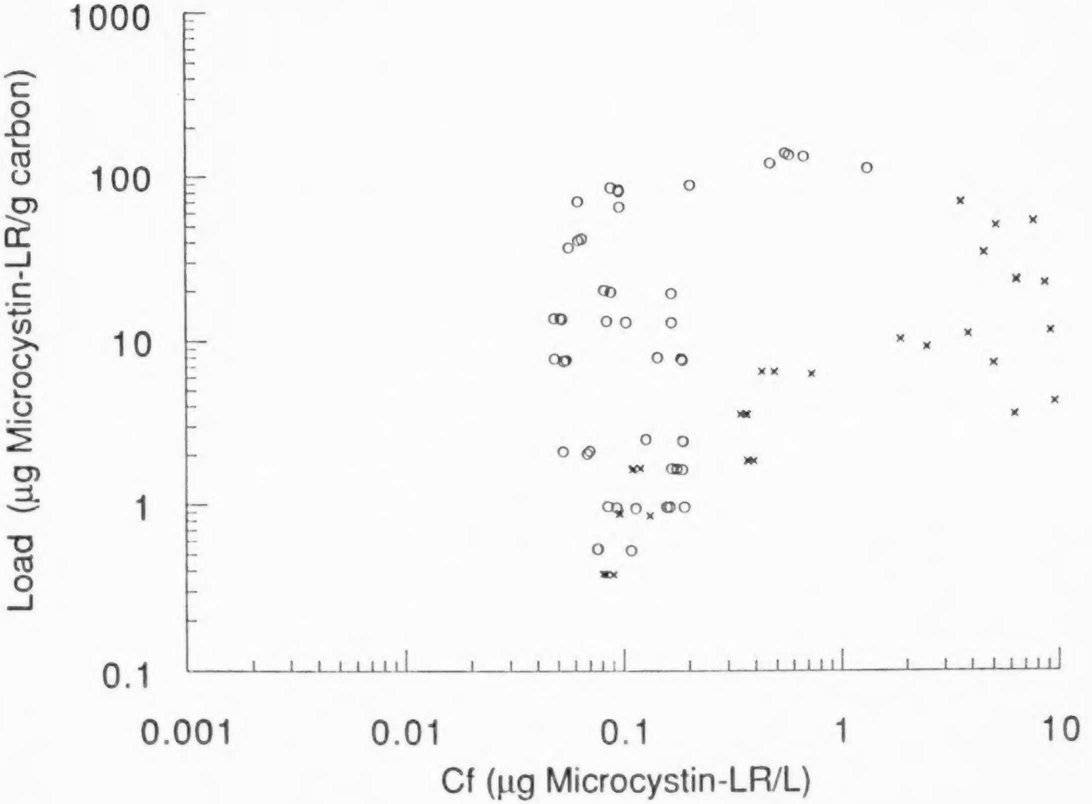


Figure 4.18 Comparison of crushed and uncrushed pre-loaded carbon. The uncrushed carbon data is indicated by "x", and the crushed carbon data by "o". Both isotherms were determined in lab grade water pH 7.8, with pre-loaded carbon removed from the Ferintosh GAC bed.

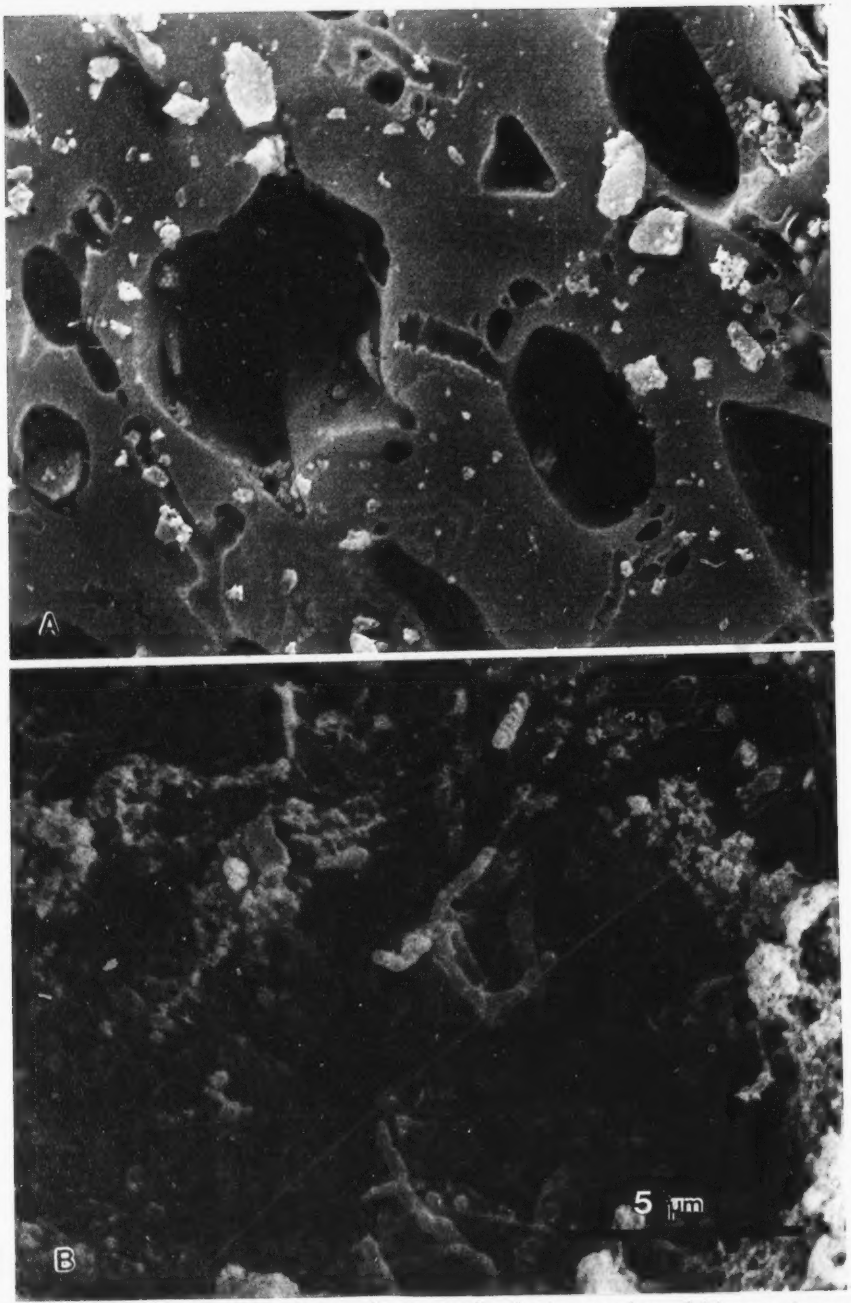


Figure 4.19 Scanning electron micrograph photographs of Haycarb carbon. A) Virgin Haycarb carbon used in the virgin adsorption isotherm and competitive adsorption isotherm. B) Pre-loaded Haycarb carbon removed from the Ferintosh treatment facility GAC bed.

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5.0 SUMMARY AND CONCLUSIONS.

Microcystin-LR was positively identified in the drinking water at the Ferintosh treatment facility in October 1992. The concentration of microcystin-LR was well below the detection limit of HPLC/UV and would not have been distinguished from background organic matter without detection by the protein phosphatase bioassay. The protein phosphatase bioassay was successfully applied to quantify microcystin in both raw and treated water at environmental concentrations. Microcystin-LR was quantified in the drinking water at concentrations as low as 0.1 µg/L microcystin-LR. The protein phosphatase bioassay represents a means of identifying and quantifying the presence of microcystin toxin in drinking water before concentrations increase to hazardous levels. Therefore the health risk associated with drinking water tainted with cyanobacteria can be minimized and the occurrence of future illnesses can be prevented.

Analysis of the Ferintosh treatment facility showed that conventional treatment processes remove some microcystin-LR from drinking water. A greater removal of the microcystin toxins can be achieved from drinking water if GAC filtration or PAC application is used in conjunction with conventional treatment. Both treatment plants, Ferintosh and Camrose, removed 70 to 80% of the raw water microcystin-LR with conventional processes and GAC or PAC respectively.

The adsorption isotherms conducted with NOM pre-loaded carbon or in the presence of NOM with virgin carbon were not significantly different. A similar carbon capacity for adsorption of microcystin-LR was observed under both conditions. In general, microcystin toxin behaves similarly to algal organic matter in water treatment processes. Improvement in the removal of algal organic matter in treatment processes will probably improve the removal of the microcystin toxins.

The major finding of this study was that in both treatment facilities, a residual concentration of microcystin-LR was observed in the finished water. In both the Ferintosh and Camrose drinking water, the residual concentration of microcystin was approximately 0.1 to 0.5 μg/L microcystin-LR. There was no apparent, community-wide incidence of acute human illness sufficient to be reported to public health authorities in either Ferintosh or Camrose during the course of the study. These data suggest that the acute incidents of human illness related to cyanobacterial toxins (see chapter two), likely occurred at higher concentrations of toxin in the drinking water. The residual concentration of microcystin in the drinking water implies that consumers will be chronically exposed to microcystin-LR for the duration of the algal bloom season.

6.0 FUTURE RESEARCH

6.1 Toxicology of Microcystin-LR

The effect of long term chronic exposure to microcystin-LR has not been thoroughly investigated. There has been only one study concerning the effect of chronic oral exposure to microcystin in water (Falconer et al. 1988). Mice were fed sub-lethal amounts of a *Microcystis* extract in their drinking water up to one year. Some mice developed damage in the liver but only at the high dosage levels. There were no effects on fertility, embryonic mortality, or teratogenicity observed other than reduced brain size in 10% of the neonatal mice when the *Microcystis* extract was given to both male and female parents starting 17 weeks prior to mating and through mating. The concentration of the microcystin(s) in the extract was not determined. Therefore research into the effects of chronic exposure to microcystins is needed.

Microcystin-LR has been shown to be a powerful liver tumor promoter (Nishiwaki-Matsushima et al. 1992). There has not been a high incidence of liver cancer in Alberta and liver cancer is the least frequently diagnosed tumour with only 35 cases diagnosed in 1990 (Berkel et al. 1992). Of course, only a small population of Albertans are exposed to microcystin-LR in their drinking water and they would not be expected to make a substantial contribution to the provincial rates. However, in Quidong County, China, a high incidence of liver cancer has been observed and was related to the source of drinking water (Delong 1979, Shun-Zhang 1989). It has been suggested that the microcystin toxin may be a possible cause (Fujiki 1991, Nishiwaki-Matsushima et al. 1992). A literature study of the research has led to an hypothesis for the role of microcystin in the high incidence of liver cancer in China.

Examination of the hepatocellular carcinomas in Quidong County has led to the observation that a tumor supressor protein, p53, contained a mutation at the same location in eight of sixteen tumor DNA samples (Hsu et al. 1991, Hollstein et al. 1991). The mutation results in a change in one nucleic acid in the DNA of the p53 gene. The nucleic acid change is the same as changes caused by aflatoxin B1 (Hsu et al. 1991, Wogan 1992). Aflatoxin B1 is a potent chemical carcinogen and mutagen in many animal species and like microcystin distributes primarily to the liver (Ewaskiewicz et al. 1991, Larsson and Tjalve 1993). Aflatoxin B1 has been quantified in the environment in Quidong (Shun-Zhang 1989) and discussed as a possible cause of the liver cancer but dismissed because it does not corroborate the geographical distribution of liver cancer in Quidong county (Delong 1979, Shun-Zhang 1989). The result of the mutation in the DNA is replacement of an arginine by a serine residue (Hsu et al. 1991, Hollstein et al. 1991). Serine residues are capable of being phosphorylated by protein kinase C and dephosphorylated by PP1 or PP2A. If the serine residue is phosphorylated, the presence of microcystin in the liver would inhibit the ability of PP1 or PP2A from dephosphorylating the serine residue. This could lead to inactivation of the p53 tumor suppressor and thereby cause the observed hepatocarcinoma in a mechanistic manner suggested by (Fujiki 1991). This provides the basis for the scenario that aflatoxin B1 acts as the initiator, causing a mutation in the p53 gene resulting in a protein kinase C phosphorylation site. Microcystin acts as a promoter by inhibition of PP1 and PP2A, causing phosphorylation and inactivation of the mutant p53 gene product, the tumor suppressor protein p53.

A second mechanism that may involve microcystin is enhancement of dioxin toxicity. The beginning of the unprecedented increase in liver cancer in Quidong can be traced to approximately the same time that the use of organo-chlorine pesticides, like DDT began to be used (Delong 1979, Shun-Zhang 1989). Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been shown to be produced during pesticide production and is the toxic component of agent orange (Bombick et al. 1985, Tritscher et al. 1992). Dioxin, like microcystin and aflatoxin, distributes primarily to the liver (Lakshmanan et al. 1986). The pathway of dioxin toxicity is dependent on protein kinase C activation (Bombick et al. 1985, Carrier et al. 1992, Berghard et al. 1993). Inhibition of PP1 and PP2A by microcystin results in an apparent activation of protein kinase C. Therefore in the presence of microcystin, the sensitivity to dioxin may be enhanced. The presence of aflatoxin B1, dioxin, and microcystin in the Quidong environment, the distribution of all the toxins to the liver, and the similarities in the mechanisms of toxicty suggest that they may be involved the high rate of liver cancer.

6.2 WATER TREATMENT OF MICROCYSTIN TOXINS.

The PP bioassay was shown to be effective at quantifying microcystin toxins at environmentally relevant concentrations. The PP bioassay measures the total concentration of microcystins in the water as a whole. Different microcystin analoques will presumably have varying response in the PP bioassay and the inhibition of PP by microcystin-LR and microcystin-RR have been shown to be different (Eriksson et al. 1990). Therefore the effect of varying concentrations of different microcystin analogues on the response of the PP bioassay should be evaluated.

The concentration of microcystin in the raw water at Ferintosh was observed to fluctuate during the day and appeared to be highest in the morning and evening on October 1. The toxin concentration of the bloom has been observed to vary over a short period of time and spatially within the bloom itself (Carmichael and Gorham 1981) and this may explain the observations. However, the release of amino acids from phytoplankton has also been observed to fluctuate through the day and has been observed to be the highest in the morning and evening (Jorgensen 1987). Ferintosh treatment facility does not draw raw water continually but operates on a need basis. Therefore a detailed analysis of the diel variations in microcystin concentration in the raw water would be very beneficial in determining the best time for drawing raw water.

The most distinguishing feature of the treatment profiles at Ferintosh was the constant residual concentration in the treated water. The concentration of microcystin leaving the GAC filter was constant despite variations in the influent concentration. The break in linearity observed in the adsorption isotherms supported the observation. A detailed time analysis of microcystin levels in the influent and effluent of the GAC filter might provide further insight into the reasons for the observations. The break in linearity in the adsorption isotherms was related to the equlibrium concentration, the presence of the bio-film and pre-loaded NOM, and competition by NOM. The effect of each of these variables could be investigated further to determine the mechanisms involved.

The residual concentration of microcystin-LR in the raw water at both Ferintosh and Camrose indicates that new treatment methods need to be evaluated if lower microcystin concentrations are desired. One process combination that may be beneficial is ozone and BGAC filtration (biological granular activated carbon). The specific reaction of ozone and microcystin has not been investigated but ozone appeared to remove microcystin from drinking water in treatment experiments (Himberg et al. 1989). Ozone may also improve the biodegradability of microcystin and competing algal organic matter. This may result in an increase in removal of microcystin by activated carbon.

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APPENDIX

Appendix 1.1 Virgin Haycarb carbon isotherm data.

Cf	Load	Estimated Y_i		Predicted Y_i	
(µg/L)	(µg MC-LR/g)	Upper-95%	Lower-95%	Upper-95%	Lower-95%
0.008	0.3	0.43	0.24	0.89	0.12
0.01	0.31	0.54	0.31	1.13	0.15
0.011	0.39	0.6	0.35	1.27	0.17
0.011	0.39	0.58	0.34	1.23	0.16
0.012	0.38	0.67	0.4	1.42	0.19
0.013	0.52	0.72	0.44	1.54	0.2
0.013	0.79	0.68	0.41	1.44	0.19
0.014	0.52	0.77	0.47	1.65	0.22
0.016	0.52	0.85	0.53	1.84	0.25
0.016	0.72	0.87	0.55	1.88	0.25
0.016	0.72	0.87	0.55	1.89	0.25
0.017	0.52	0.88	0.56	1.92	0.26
0.0267	1.57	1.4	0.95	3.12	0.42
0.0286	1.4	1.49	1.02	3.34	0.46
0.0379	1.39	1.97	1.39	4.47	0.61
0.074	2.82	3.87	2.82	8.9	1.23
0.076	2.78	4	2.92	9.2	1.27
0.077	2.8	4.07	2.96	9.35	1.29
0.092	4.42	4.92	3.56	11.28	1.55
0.095	4.4	5.08	3.68	11.65	1.6
0.099	4.5	5.31	3.83	12.15	1.67
0.1	10.67	5.34	3.85	12.22	1.68
0.101	4.49	5.44	3.92	12.45	1.71
0.102	11.95	5.46	3.93	12.49	1.72
0.104	12.58	5.57	4.01	12.74	1.75
0.108	10.54	5.81	4.17	13.27	1.83
0.11	4.5	5.94	4.26	13.55	1.86
0.15	2.51	8.32	5.8	18.77	2.57
0.2537	13.37	14.9	9.68	32.69	4.41
0.2617	14.55	15.43	9.97	33.78	4.55
0.2624	14.53	15.47	9.99	33.87	4.57
0.2729	12.88	16.17	10.38	35.32	4.75
0.44	13.55	27.52	16.28	58.27	7.69
0.48	13.4	30.72	17.84	64.6	8.48
0.488	74.47	31.17	18.06	65.49	8.6
0.49	16.92	31.59	18.26	66.32	8.7
0.542	39.7	35.16	19.97	73.34	9.57
0.621	14.93	41.06	22.71	84.84	10.99
0.653	14.53	43.45	23.8	89.47	11.56

Appendix 2.1 Competitive isotherm data; Virgin Haycarb carbon and Ferintosh water.

Cf	Load	Estimated Yi		Predicted Yi	
(µg/L)	(µg MC-LR/g)	Upper-95%	Lower-95%	Upper-95%	Lower-95%
0.04	0.29	0.39	0.15	0.68	0.09
0.04	0.39	0.41	0.18	0.74	0.1
0.05	0.38	0.51	0.3	1.01	0.15
0.05	0.39	0.53	0.32	1.05	0.16
0.05	0.57	0.51	0.3	1.01	0.15
0.05	0.23	0.53	0.32	1.05	0.16
0.05	0.24	0.51	0.3	1.01	0.15
0.05	0.3	0.47	0.25	0.9	0.13
0.05	0.3	0.57	0.35	1.14	0.17
0.06	1.1	0.81	0.45	1.56	0.23
0.06	1.11	0.98	0.49	1.82	0.26
0.07	1.16	1.13	0.51	2.04	0.28
0.07	0.58	1.3	0.53	2.28	0.3
0.07	0.57	1.24	0.53	2.2	0.3
0.12	3.8	5.98	2.85	10.84	1.57
0.12	3.64	5.88	2.78	10.62	1.54
0.13	3.66	6.06	2.92	11.01	1.6
0.2	12.67	7.35	3.95	13.83	2.1
0.27	6.4	8.36	4.79	16.09	2.49
0.31	6.16	8.88	5.23	17.26	2.69
0.31	6.38	8.86	5.22	17.22	2.68
0.79	9.39	13.91	9.13	28.14	4.51
0.87	6.94	14.64	9.62	29.63	4.75
0.97	5.88	15.53	10.19	31.42	5.04
1.33	33.7	18.55	11.97	37.3	5.96
2.89	21.35	29.86	17.15	57.52	8.9
2.94	32.35	30.16	17.27	58.04	8.97
3.15	30.82	31.54	17.8	60.4	9.3
3.65	25.66	34.66	18.96	65.65	10.01
5.1	37.96	43.12	21.83	79.62	11.82
5.11	31.04	43.17	21.85	79.7	11.83
5.53	19.59	45.48	22.57	83.45	12.3

Appendix 3.1 Pre-loaded isotherm data; Pre-loaded Haycarb carbon and lab-grade water.

Cf	Load	Estimated Yi		Predicted Yi	
(µg/L)	(µg MC-LR/g)	Upper-95%	Lower-95%	Upper-95%	Lower-95%
0.08	0.38	0.8	0.26	1.48	0.14
0.08	0.38	0.77	0.23	1.4	0.13
0.09	0.38	0.91	0.4	1.82	0.2
0.1	0.88	1.03	0.5	2.14	0.24
0.1	0.88	1.01	0.49	2.09	0.23
0.11	1.62	1.55	0.71	3.17	0.35
0.11	1.66	1.61	0.72	3.26	0.36
0.12	1.66	2.22	0.81	4.23	0.43
0.13	0.86	3.6	0.91	6.26	0.53
0.34	3.59	6.1	1.84	20.71	0.54
0.36	3.58	6.23	1.92	21.3	0.56
0.37	1.85	6.28	1.96	21.59	0.57
0.37	1.86	6.27	1.95	21.51	0.57
0.37	3.57	6.27	1.95	21.51	0.57
0.39	1.85	6.41	2.05	22.21	0.59
0.43	6.59	6.62	2.2	23.22	0.63
0.49	6.55	6.95	2.44	24.84	0.68
0.73	6.37	8.14	3.32	30.76	0.88
1.87	10.45	12.77	6.23	51.69	1.54
2.48	9.42	15.13	7.29	60.94	1.81
3.55	71.65	19.19	8.69	75.46	2.21
3.84	11.31	20.28	9.01	79.14	2.31
4.55	35.28	22.91	9.7	87.78	2.53
5.03	7.48	24.66	10.12	93.36	2.67
5.15	51.56	25.1	10.22	94.76	2.71
6.25	3.62	29.04	11.04	106.89	3
6.35	24.22	29.4	11.11	108	3.02
6.4	23.9	29.58	11.14	108.54	3.04
7.64	55.08	33.92	11.92	121.41	3.33
8.62	22.97	37.29	12.47	131.14	3.55
9.17	11.88	39.15	12.76	136.46	3.66
9.58	4.34	40.53	12.96	140.36	3.74

Appendix 4.1 Virgin carbon isotherm data for Norit 0.8 supra and Calgon F-100 carbon

Norit Carbon

Cf (µg/L)	Load (µg MC-LR/g)
0.08	0.358
0.0915	0.35
0.0889	0.362
0.1253	0.69
0.1168	0.679
0.136	0.672
0.21	36.572
0.544	17.268
0.315	31.096
0.068	13.127
0.105	11.638
0.13	7.091

Calgon F-100

Cf (µg/L)	Load (µg MC-LR/g)
0.0731	0.364
0.0684	0.366
0.0642	0.371
0.0839	0.706
0.0781	0.706
0.0745	0.7
0.317	57.127
0.285	52.919
0.269	54.569

Appendix 5.1 Competitve carbon isotherm data for Norit 0.8 supra and Calgon F-100.

Norit Carbon

Cf (µg/L)	Load (µg MC-LR/g)
0.0373	0.303
0.0425	0.3
0.0816	0.581
0.0809	0.567
0.0904	0.572
0.27	13.15
0.15	6.89
0.26	6.75

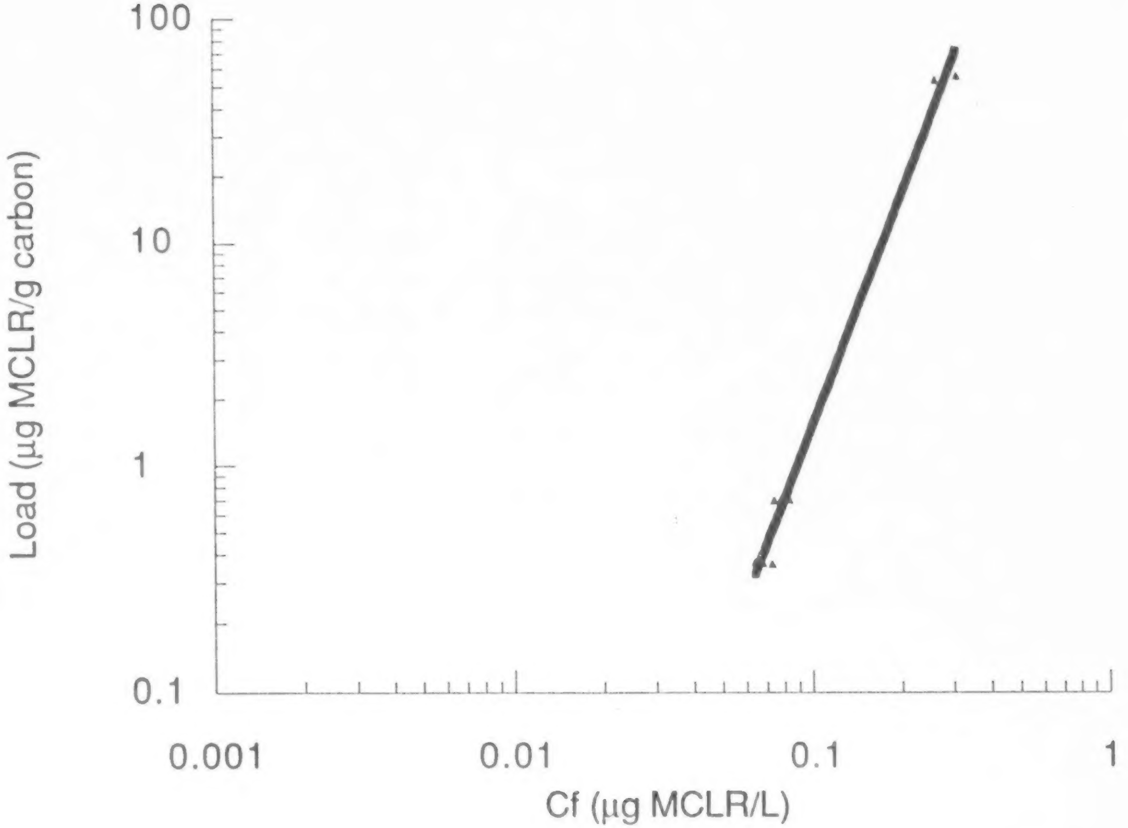
Calgon F-100

Cf (µg/L)	Load (µg MC-LR/g)
0.0164	0.306
0.0174	0.304
0.0561	0.591
0.0456	0.586
0.0442	0.574
0.33	6.019
0.35	5.941
0.36	6.16
0.66	11.087
0.61	11.88
0.46	12.847

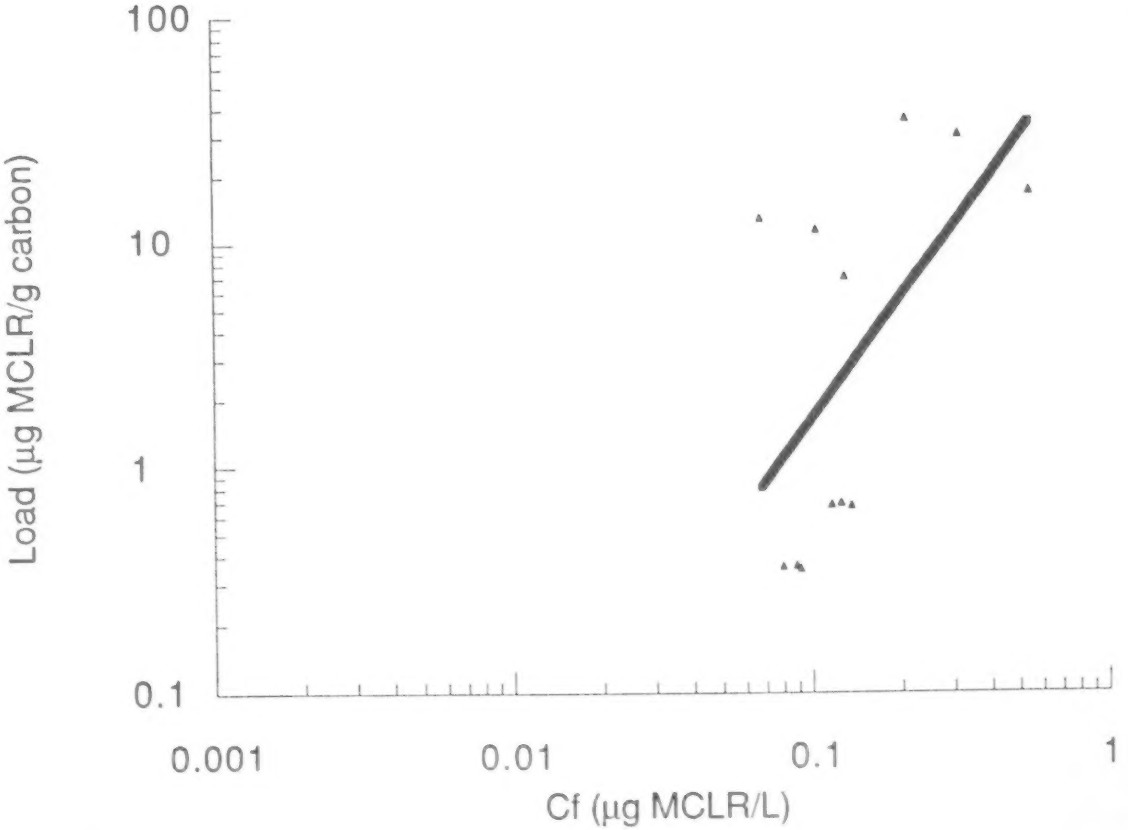
Appendix 6.1 Biodegradation Analysis of Microcystin-LR by the Biofilm on Pre-loaded Haycarb.

Date	Biodegradation Samples (µg/L microcystin-LR)			Control Samples - Sodium Azide Present (µg/L microcystin-LR)		
	1	2	3	4	5	6
31-Oct	0.39	0.49	-	0.62	-	0.94
1-Nov	0.44	0.35	0.43	0.52	0.6	0.77
4-Nov	0.38	0.36	0.47	1.06	0.8	0.72
16-Nov	0.52	0.57	0.5	0.96	0.91	0.86
1-Dec	0.66	0.5	0.58	0.55	0.77	1.02
4-Dec	0.39	0.43	0.4	0.52	0.56	0.56

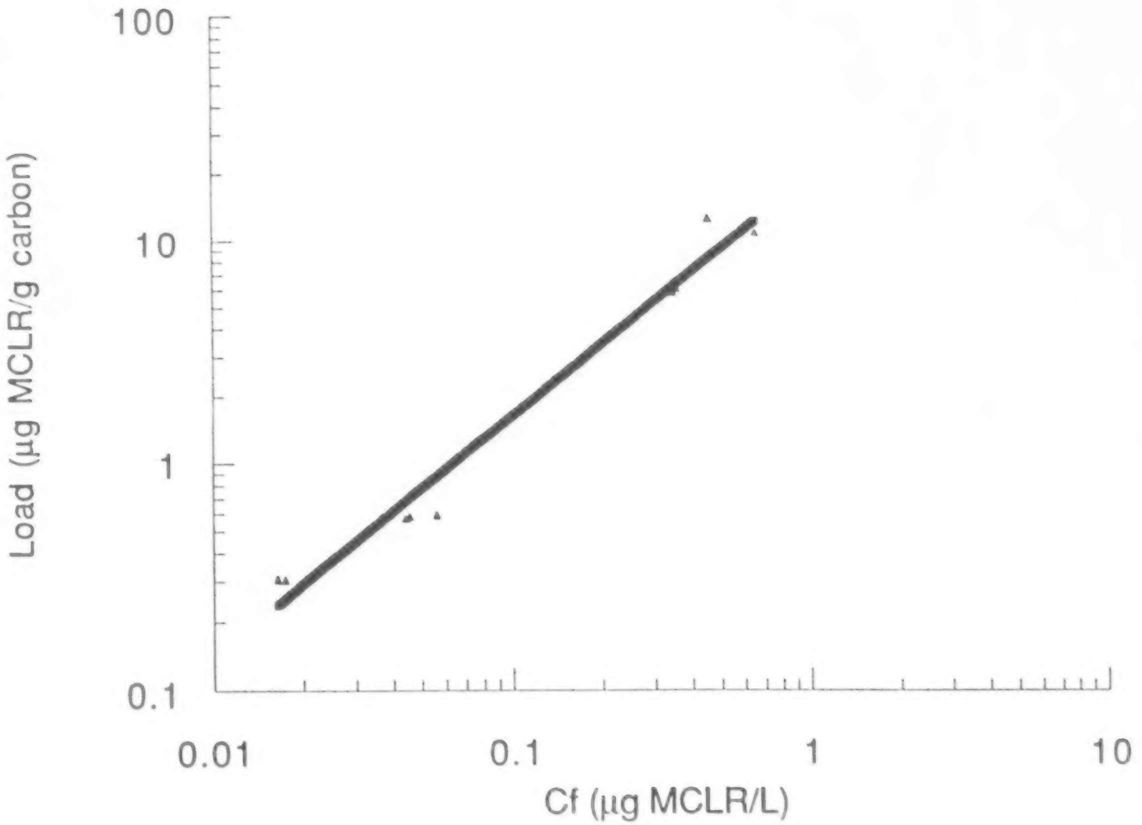
Date	Biodegradation Samples			Control Samples - Sodium Azide Present		
	1	2	3	4	5	6
31-Oct	0.25	0.3	-	0.45	-	0.75
1-Nov	0.37	0.34	0.42	0.35	0.36	0.38
4-Nov	0.25	0.3	0.25	0.54	0.45	0.46
16-Nov	0.29	0.36	0.33	0.45	0.42	0.4
1-Dec	0.42	0.36	0.29	0.41	0.42	0.46
4-Dec	0.24	0.26	0.22	0.28	0.33	0.34



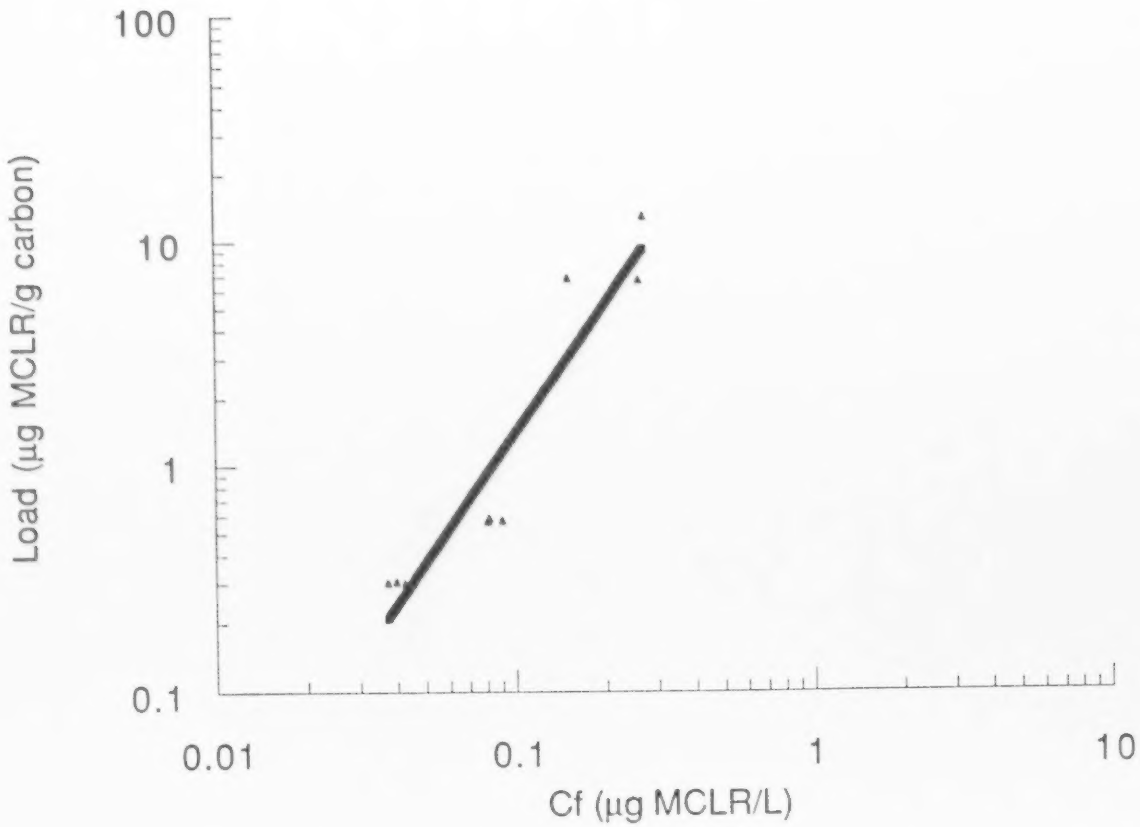
Appendix 7.1 Virgin Carbon Isotherm for Microcystrin-LR Prepared with Calgon F-100, pH 7.8.



Appendix 7.2 Virgin Carbon Isotherm for Microcystin-LR Prepared with Norit 0.8 Supra, pH 7.8.



Appendix 8.1 Competitive Sorption Isotherm for Microcystin-LR Prepared with Calgon F-100, TOC= 17 mg/L, pH 7.8.



Appendix 8.2 Competitive Sorption Isotherm for Microcystin-LR Prepared with Norit 0.8 Supra, TOC= 17 mg/L, pH 7.8.